UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

Departamento de Química Agrícola y Bromatología

Development of sustainable novel foods based on coffee by-products for chronic diseases

Desarrollo de nuevos alimentos sostenibles basados en subproductos de café para enfermedades crónicas

Nuria Martínez Sáez

Madrid, Mayo 2017

INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

María Dolores del Castillo, Dra. en Ciencia y Tecnología de los Alimentos e Investigadora Científica de Instituto de Investigación en Ciencias de la Alimentación (CIAL) del Consejo Superior de Investigaciones Científicas (CSIC), y **María Ángeles Martín Cabrejas**, Profesora Titular de Universidad del Departamento de Química Agrícola y Bromatología de la Facultad de Ciencias de la Universidad Autónoma de Madrid (UAM).

Informan:

Que el presente trabajo titulado **"Development of sustainable novel foods based on coffee by-products for chronic diseases"** ("Desarrollo de nuevos alimentos sostenibles basados en subproductos de café para enfermedades crónicas") constituye la memoria que presenta **Nuria Martínez Sáez,** Licenciada en Ciencia y Tecnología de los Alimentos por la UAM, para optar al GRADO DE DOCTORA. La presente memoria ha sido realizada bajo su dirección en el Departamento de Bioactividad y Análisis de Alimentos del CIAL (UAM-CSIC) y reúne las condiciones necesarias para su presentación y defensa.

Y para que conste a los efectos oportunos, firmamos el presente certificado,

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C/ Nicolás Cabrera, 9.
Campus de la Universidad
Autónoma de Madrid
28049 Madrid

A mi punto cardinal,
cómplice y mitad… a ti
abuelo,

Acknowledgements

"If I have seen further it is by standing upon the shoulders of giants"

Isaac Newton

Grants and Funding

The present study has been funded by the following projects:

1. Nuevos ingredientes antiglicantes: Mecanismos de acción y aplicaciones en alimentación y salud (NATURAGE, AGL2010-17779) funded by the Interministerial Commission of Science and Technology (CICYT).

2. Proteínas de soja, estructura, interacciones y funciones (IT2009-0087) funded by the Ministry of Economy and Competitiveness.

3. Producción y consumo sostenibles del café: validación de subproductos como ingredientes alimentarios (SUSCOFFEE, AGL2014-57239-R) funded by the Ministry of Economy and Competitiveness.

Nuria Martínez Sáez has received financial support from the “Programa Propio de Ayudas de Formación de Personal Investigador (FPI-UAM) de 2013” funded by the Autonomous University of Madrid (UAM).

Mobility awards have also been granted to Nuria Martínez Sáez, “Convocatoria de Estancias Breves en España y en el Extranjero del Programa Propio de Contratos Predoctorales para Formación de Personal Investigador (FPI-UAM)” in 2014 and 2016, funded by the Autonomous University of Madrid (UAM).

List of publications

Very related to the PhD thesis:

Patent

- del Castillo, M. D.; **Martinez-Saez, N**.; Ullate, M. Formulación alimentaria que comprende marros de café y sus aplicaciones, 2014, WO2014128320 A1 (***Annex 1***).

Research articles

- **Martinez-Saez, N.**; Ullate, M.; Martin-Cabrejas, M. A.; Martorell, P.; Genovés, S.; Ramon, D.; del Castillo, M. D. A novel antioxidant beverage for body weight control based on coffee silverskin. Food Chem. 2014, 150, 227–234.
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- del Castillo, M. D.; Iriondo-DeHond, A.; Fernandez-Gomez, B.; **Martinez-Saez, N.**; Rebollo-Hernanz, M.; Martín-Cabrejas, M. A.; Farah, A. Coffee antioxidants in chronic diseases. In Coffee: Chemistry, Quality and Health Implications; Farah, A., Ed.; RSC Publishing Inc.; In press.

Related to the PhD thesis:

- del Castillo, M. D.; **Martinez-Saez, N**.; Amigo-Benavent, M.; Silvan, J. M. Phytochemomics and other omics for permitting health claims made on foods. Food Res. Int. 2013, 54, 1237–1249 (***Annex 2).***
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"From error to error, one discovers the entire truth"

Sigmund Freud

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Abbreviations

ABBREVIATIONS

5-HT	Serotonin
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACSE	Arabica coffee silverskin extract
ADI	Acceptable Daily Intake
AGEs	Advanced glycation end products
BMI	Body mass index
BSA	Bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CFB	Coffee fibre-containing biscuit
CGA	Chlorogenic acid
CML	Carboxymethyl-lysine
CQA	Caffeoylquinic acid
CS	Coffee silverskin
CSE	Coffee silverskin extract
CZE	Capillary zone electrophoresis;
DCQA	Dicaffeoylquinic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
FFAs	Free fatty acids
FOS	Fructo-oligosaccharides
FRAP	Ferric reducing antioxidant power
FSGs	Foods for Specific Groups
GI	Glycaemic index
GL	Glycaemic load
GLP-1	Glucagon-like peptide-1
HbA1C	Glycated haemoglobin
HFCS	High fructose corn syrup
HMF	5-hydroxymethylfurfural
HPLC	High-performance liquid chromatography
LES	Low-energy sweeteners
MGO	Methylglyoxal

MR	Maillard reaction
MRPs	Maillard reaction products
NBT	Nitroblue tetrazolium
NSP	Non-starch polysaccharides
ORAC	Oxygen radical absorbance capacity
RCSE	Robusta coffee silverskin extract
RDS	Rapidly digestible starch
SCB	Sucrose-containing biscuit
SCG	Spent coffee grounds
SDS	Slowly digestible starch
SENC	Spanish Society of Community Nutrition
T1D	Type 1 diabetes
T2D	Type 2 diabetes
WHO	World Health Organization

Summary

SUMMARY

At present, the global obesity epidemic is showing no signs of abating, which is fuelling an explosion in numbers of type 2 diabetes (T2D) worldwide. In order to control these two associated diseases, lifestyle modification, which encompasses diet, physical activity, and healthy habits, might be useful. Several studies have focused the attention on modifying the main nutrients of the diet to reduce body weight and to enhance blood glycaemia among other metabolic biomarkers. The use of phytochemicals and bioactive compounds obtained from vegetable sources might be a favourable approach to reduce the risk of metabolic chronic diseases; however, it is still a challenge to face. Coffee silverskin (CS) and spent coffee grounds (SCG) are very abundant coffee wastes worldwide generating global environmental problems. They are natural sources of several bioactive compounds with potential to reduce the risk of chronic non-communicable diseases, such as chlorogenic acid (CGA), caffeine and dietary fibre. The widespread interest in select foods that might promote health has resulted in the use of functional foods to provide specific health benefits beyond basic nutrition. Therefore, these coffee wastes may become sustainable high value-added ingredients of interest for the management of obesity and T2D.

The present PhD thesis aimed to produce sustainable high sensorial quality beverage and foods, for reducing the risk of chronic metabolic diseases, by means of employing as novel ingredients coffee by-products (CS and SCG) and their derivatives, in combination with other healthy ingredients. In addition, the present investigation search for studying the impact of the digestive process of these functional foods containing coffee by-products, on the release of satiety hormones; as well as, the implication of this physiological event on the formation of potential harmful compounds such as Maillard reaction products (MRPs).

For the first time, the application of CS for the elaboration of a high sensorial quality and sustainable antioxidant beverage was proposed. CS extract (CSE)-containing beverages possessed suitable nutritional quality regarding the content of soluble dietary fibre, proteins and glycaemic sugars. Physiologically active concentrations of bioactive compounds such as CGA and caffeine were detected in the beverages, which have shown to contribute in the reduction of fat deposits in the worm model of *Caenorhabditis elegans*. The beverage prepared with Robusta CSE (RCSE), at 10 mg/ml, was the most effective dose, achieving a body fat reduction of 24%. Moreover, this beverage presented similar effect on body fat accumulation to that found in a commercial decaffeinated green coffee supplement. Furthermore, the feasibility of the combined use of non-nutritive sweetener and CS, in order to achieve healthier, nutritious and good quality biscuits, was also investigated. CS was used as a natural colouring and as a source of dietary fibre, leading to full recovery of this coffee

waste. The texture and nutritional properties of the biscuits were also improved, and the typical golden colour expected of the baked products was provided. The neo-contaminant hydroxymethylfurfural (HMF) was greatly reduced and no bioaccessible acrylamide was detected in the digests of the new biscuits.

On the other hand, this is the first time that SCG from the instant coffee process have been proposed as sustainable natural source of antioxidant insoluble dietary fibre. Its application as food ingredient in bakery products was patented (WO2014128320 A1) during the development of the PhD thesis. The coffee fibre was exhausted of sugars and processing contaminants, and presented small levels of free bioactive compounds (CGA and caffeine), as a consequence of the extraction process in the preparation of soluble coffee beverage. The coffee fibre was stable to the baking and to the abiotic gastrointestinal digestion *in vitro*. All these findings make the coffee fibre valid for its use as food ingredient. Non-nutritive sweeteners and fructooligosaccharides (FOS) were included in biscuits, which contain antioxidant coffee fibre, to obtain bakery products that meet consumers' preferences. No significant differences were found with those commercial biscuits presenting high consumer adhesion and similar characteristics in terms of non-nutritive sweeteners and dietary fibre content. The novel coffee fibre-containing biscuit (CFB) presented reduced levels of neo-contaminants, in particular 67% less of acrylamide (166 µg acrylamide/kg) than the indicative values in biscuits established by the European Commission. A 4-fold decrease in the compounds associated to diabetes, such as advanced glycation end products (AGEs), was also detected compared to a sucrose-containing biscuit (SCB). The digest obtained from simulated human digestion of the CFB presented significantly lower glycaemic sugar content (60.6 mg/g) and a higher antioxidant capacity (15.1 mg CGA/g) than SCB. Antidiabetic and satiety effects were described for the CFB. The bioaccessible fraction of the CFB digest presented significantly higher inhibitory effect on α-glucosidase activity (IC50 = 3.3 mg/ml) than the SCB (IC50 = 6.2 mg/ml), and promoted the release of satiety hormones, serotonin and glucagon-like peptide-1 (GLP-1) by Caco-2 and HuTu-80 cells, respectively (355% and 278%), in the same order of magnitude as the SCB.

The formation of MRPs during digestive process of simplified meal systems was for the first time examined. Our results support the formation of non-fluorescent AGEs associated to the pathogenesis of diabetes during the digestion of model systems mimicking sugar-containing and average meals, respectively. Decrease of lysine (11.7-34%), arginine (24-35%) and other amino acids occurred during the digestion process affecting their bioavailability and involving health implications. Fructosamine (42.6 and 332.9 µg/ml) and fluorescent adducts (22270 and 9283 RFU) were detected in digests of those meals containing high-fructose corn syrup (HFCS) and starch,

respectively. Carboxymethyl-lysine (CML) (5 µg/ml) and methylglyoxal (MGO)-derivative AGEs (12.2 µg/ml) were found in the meal systems composed of fructose.

In conclusion, the findings derived from the present PhD thesis demonstrate the feasibility of using coffee by-products, CS and SCG, in the development of novel sustainable beverages and foods with enhanced technological, nutritional and sensorial quality. For the first time, technological strategies were achieved for valorisation of coffee by-products into novel health promoting food ingredients, avoiding the production of new industrial wastes. Scientific evidences regarding the potential of the sustainable beverages and foods for reducing the risk of obesity and T2D were obtained. The novel beverages and foods decreased body fat deposits *in vivo*, induced the release of satiety gut hormones related to the feeling of fullness *ex vivo* and exhibited inhibitory effect on α-glucosidase enzyme *in vitro*. Finally, preliminary evidence on the impact of the digestive process of an average and sugary meal systems suggested the formation of compounds of interest in health and diseases in the intestinal lumen.

Resumen

RESUMEN

Actualmente, la epidemia mundial de obesidad no muestra signos de disminución, lo cual está conduciendo a una explosión de diabetes tipo 2 en todo el mundo. Con el fin de controlar estas dos enfermedades asociadas, la modificación del estilo de vida, la cual incluye cambios en la dieta, actividad física y hábitos saludables, parece ser de utilidad. Varios estudios han centrado la atención en la modificación de los principales nutrientes de la dieta con el fin de reducir el peso corporal y mejorar la glucemia sanguínea entre otros biomarcadores metabólicos. El uso de fitoquímicos y compuestos bioactivos obtenidos a partir de fuentes vegetales podría ser una estrategia adecuada para reducir el riesgo de enfermedades crónicas metabólicas; sin embargo, sigue siendo un reto a abordar. La cascarilla y los posos de café, son abundantes subproductos del café que generan problemas medioambientales a nivel mundial. Son fuentes naturales de algunos compuestos bioactivos con potencial para reducir el riesgo de enfermedades crónicas no transmisibles, como el ácido clorogénico, la cafeína y la fibra dietética. El interés creciente por alimentos que puedan promover la salud, ha resultado en la aparición de los alimentos funcionales, con el fin de proporcionar así beneficios específicos para la salud, más allá de la propia nutrición básica. Por tanto, estos residuos de café podrían convertirse en ingredientes sostenibles de interés con alto valor añadido para el control de la obesidad y la diabetes tipo 2.

La presente tesis doctoral tiene como objetivo elaborar bebidas y alimentos sostenibles de alta calidad sensorial, con el fin de reducir el riesgo de las enfermedades crónicas metabólicas, mediante el empleo de subproductos del café (cascarilla y posos de café) y sus derivados, en combinación con otros ingredientes saludables. Además, la presente investigación busca estudiar el impacto del proceso digestivo de los alimentos funcionales elaborados, que contienen estos subproductos del café, sobre la liberación de hormonas de saciedad, así como la implicación de este evento fisiológico en la formación de posibles compuestos nocivos, tales como los productos de la reacción de Maillard (MRPs, de sus siglas en inglés).

Por primera vez se ha propuesto la aplicación de cascarilla de café, para la elaboración de una bebida antioxidante, sostenible, y de alta calidad sensorial. Las bebidas preparadas con extracto de cascarilla presentaron una adecuada calidad nutricional en relación al contenido de fibra dietética soluble, proteínas y azúcares glucémicos. Se encontraron concentraciones fisiológicamente activas de compuestos bioactivos en estas bebidas, tales como ácido clorogénico y cafeína, los cuales mostraron contribución sobre la reducción de depósitos de grasa en el modelo de gusano *Caenorhabditis elegans*. La dosis más eficaz la mostró la bebida preparada con extracto de

cascarilla Robusta, en concentraciones de 10 mg/ml, logrando una reducción del 24% de la grasa corporal. Además, esta bebida presentó un efecto similar sobre los depósitos de grasa corporal al mostrado por un suplemento comercial de café verde descafeinado. Además, se ha investigado también la viabilidad del empleo combinado de edulcorantes no nutritivos y cascarilla de café con el fin de lograr galletas más sanas, nutritivas y de buena calidad. La cascarilla se utilizó como colorante natural y como fuente de fibra dietética, lo que condujo a una recuperación completa de este subproducto de café. Se consiguió una mejora de la textura y propiedades nutricionales de las galletas y además se proporcionó el color dorado típico de los productos horneados. El contenido en hidroximetilfurfural se redujo considerablemente y no se detectó acrilamida bioaccesible en los digeridos de las nuevas galletas.

Por otra parte, se ha propuesto por primera vez, el uso de los posos de café procedentes del proceso de café instantáneo como fuente natural sostenible de fibra dietética antioxidante insoluble. Su aplicación, como ingrediente alimentario en productos de panadería, ha sido patentada (WO2014128320 A1) durante el desarrollo de la tesis doctoral. La fibra de café mostró estar exhausta en azúcares y contaminantes del procesado, y además presentó bajos niveles de compuestos bioactivos libres (ácido clorogénico y cafeína), como consecuencia del proceso de extracción en la preparación de la bebida de café soluble. La fibra de café presentó estabilidad al horneado y a la digestión gastrointestinal abiótica *in vitro*. Todos estos hallazgos hacen que la fibra de café sea válida para su uso como ingrediente alimentario. Edulcorantes no nutritivos y fructooligosacáridos (FOS) se incorporaron en la formulación de galletas, las cuales contenían fibra de café antioxidante, con el fin de obtener productos de panadería que satisfagan las preferencias de los consumidores. No se encontraron diferencias significativas con galletas comerciales con alta aceptación en el mercado y características similares en cuanto a edulcorantes no nutritivos y contenido en fibra dietética. La nueva galleta de fibra de café presentó niveles reducidos de neo-contaminantes, en particular un 67% menos de acrilamida (166 µg acrilamida/kg) que los valores indicativos para galletas establecidos por la Comisión Europea. También se detectó una disminución 4 veces menor en compuestos asociados a la diabetes, como son los productos avanzados de la glicación (AGEs, de sus siglas en inglés), en comparación con una galleta de sacarosa. El digerido obtenido a partir de la digestión humana simulada de la galleta de fibra de café, presentó un contenido de azúcar glicémico significativamente menor (60,6 mg/g) y una capacidad antioxidante mayor (15,1 mg CGA/g), que la galleta de sacarosa. La galleta de fibra de café mostró efectos antidiabéticos y de saciedad. La fracción bioaccesible del digerido exhibió un efecto inhibidor sobre la actividad α-glucosidasa (IC50 = 3,3 mg/ml), el cual fue significativamente mayor que la galleta de sacarosa (IC50 = 6,2 mg/ml) y además, promovió la liberación de hormonas

de saciedad tales como, serotonina y péptido similar al glucagón tipo 1, en las células Caco-2 y HuTu-80, respectivamente (355% y 278%), en el mismo orden de magnitud que la galleta de sacarosa.

Por primera vez se ha evaluado la formación de MRPs, durante el proceso digestivo a partir de sistemas de comida simplificada. Nuestros resultados apoyan la formación de AGEs no fluorescentes asociados a la patogénesis de la diabetes durante la digestión de sistemas modelo que simulan comidas con azúcar y una comida promedio, respectivamente. Se observó una disminución de lisina (11,7-34%), arginina (24-35%) y otros aminoácidos durante el proceso de digestión, afectando así su biodisponibilidad y por tanto, con implicaciones para la salud. En los digeridos de sistemas de comida con jarabe de alto contenido en fructosa o almidón, se detectaron fructosamina (42,6 y 332,9 µg/ml) y aductos fluorescentes (22270 y 9283 RFU), respectivamente. En los modelos de comida preparados con fructosa se encontraron carboximetil-lisina (CML) (5 µg/ml) y AGEs derivados de metilglioxal (12,2 µg/ml).

En conclusión, los resultados derivados de la presente tesis doctoral demuestran la viabilidad del empleo de subproductos del café, cascarilla y posos, en el desarrollo de nuevas bebidas y alimentos sostenibles, de mayor calidad tecnológica, nutricional y sensorial. Por primera vez se han logrado estrategias tecnológicas, para la revalorización de los subproductos del café, como nuevos ingredientes alimentarios que promueven la salud, y evitando así la producción de nuevos subproductos industriales. Se han obtenido además, evidencias científicas sobre el potencial de las bebidas y alimentos sostenibles, para reducir el riesgo de obesidad y diabetes tipo 2. Las nuevas bebidas y alimentos disminuyen los depósitos de grasa corporal *in vivo*, promueven la liberación de hormonas de saciedad intestinales *ex vivo* y muestran un efecto inhibidor *in vitro* sobre la enzima α-glucosidasa. Por último, la evidencia preliminar sobre el impacto del proceso digestivo de sistemas de comida azucarada y de una comida promedio, sugiere la formación en el lumen intestinal de compuestos de interés en la salud y enfermedades.

Introduction

INTRODUCTION

1. Metabolic chronic diseases: Obesity and Diabetes

Currently, chronic diseases are the leading global causes of death. It is projected to increase up to 52 million of deaths by 2030 [1]. Obesity and type 2 diabetes (T2D) have reached epidemic proportions and are worldwide health problems. These metabolic chronic diseases are caused, to a large extent, by behavioural risk factors such as changes in dietary macronutrient intake [2]. Therefore, it is possible to reduce the risk of these metabolic diseases through different strategies being the most sustainable the promotion of a healthy diet.

1.1. Obesity

Obesity is a chronic disease characterized by the expansion of adipose tissue and an inflammatory component [3]. Adipose tissue it is now recognized as an active tissue in the regulation of physiological and pathological processes, including immunity and inflammation. Adipose tissue releases a variety of adipokines, anti- or pro-inflammatory cytokines such leptin, TNF-α and interleukin [IL]-4 [4]. These pro-inflammatory molecules play an important role on the development of metabolic disease such as T2D and cardiovascular disease [5].

Obesity can be classified according to different criteria:

- By body mass index (BMI) and its related risk of mortality: class I for a BMI between 30 and 34.9 kg/m^2, class II for a BMI between 35 and 39.9 kg/m^2, and class III for a BMI ≥ 40 kg/m^2 [6]. In turn, class I obesity is associated with a “moderate risk”, class II with a “high risk”, and class III with a “very high risk” of mortality [7].
- By anatomic phenotypes, it is referred as visceral or a subcutaneous obesity depending on the degree of central (i.e. visceral) and peripheral (i.e. subcutaneous) fat. The first term indicates a major risk factor for metabolic complications while subcutaneous fat seems to be much more benign [8].
- From an etiologic standpoint obesity can be fundamentally classified as primary or secondary. Obesity presents as primary etiology, multifactorial causes involving genetics, hormones, diets, and environment [9]. Diet is an important regulatory factor on immune response and there is evidence to suggest that over-nutrition leads to immune-activation which is related to obesity [4]. A secondary obesity is that developed as a consequence of for example pharmacologic treatments.

Moreover, there is another important phenotype called "metabolically obese normal weight individuals". They are characterized by increased levels of adiposity and insulin resistance and a higher susceptibility to T2D and cardiovascular diseases [10]. Ongoing investigations reveal this phenotype is not uncommon [11], however, a consensus on the definition is lacking, and measuring the degree of insulin resistance or adiposity is not widely applicable.

Overweight and obesity have been increasing in all countries. In 2014, 39% of adults aged 18 years and older (38% of men and 40% of women) were overweight. The prevalence of obesity nearly doubled between 1980 and 2014. In fact, in 2014, 11 % of men and 15% of women were obese. Thus, more than half a billion adults worldwide are classed as obese (***figure 1***) [1].

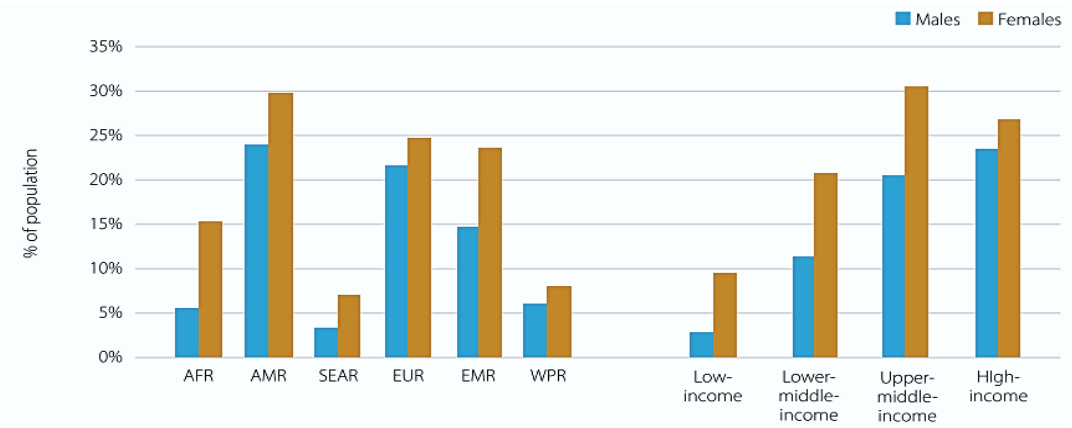


Figure 1. Age-standardized prevalence of obesity (BMI ≥ 30 kg/m^2) in adults aged 18 years and over by region and income group, comparable country estimates, 2014. [AFR=Africa, AMR=America, SEAR =South-East Asia, EUR=Europe, EMR=Eastern Mediterranean, WPR=Western Pacific] (World Health Organization (WHO), 2014) [1].

1.2. Diabetes

Diabetes mellitus is a chronic disorder characterised by major derangements in glucose metabolism and abnormalities in fat and protein metabolism [12]. Diabetes is broadly classified under two categories, type 1 (T1D) and type 2 diabetes (T2D).

- **T1D** is the chronic condition caused by the absence of insulin secretion due to either the marked inability of the β-Langerhans islet cells of the pancreas to produce insulin, then glucose accumulates in the blood serum unless insulin is supplied [13]. The insulin-dependent diabetes is inherited and usually occurs early in life. The major factor in the pathophysiology of T1D is considered to be autoimmunity, with immune response against altered β-cells [13].

- **T2D** is a progressive condition in which the body becomes resistant to the normal effects of insulin and/or gradually loses the capacity to produce enough insulin in the pancreas [13]. T2D has a different pathophysiology and etiology than T1D. T2D is a combination of low amounts of insulin production from pancreatic β-cells and peripheral insulin resistance. These two events must occur simultaneously for T2D to develop (***figure 2***) [14]. T2D is the most common type of diabetes representing 90–95% of all cases. Most patients with T2D, but not all, are overweight or obese, in fact, this excess weight itself causes some degree of insulin resistance. Nutritional intervention is a good strategy for reducing the risk of TD2 and one of the main research lines of the present PhD thesis.

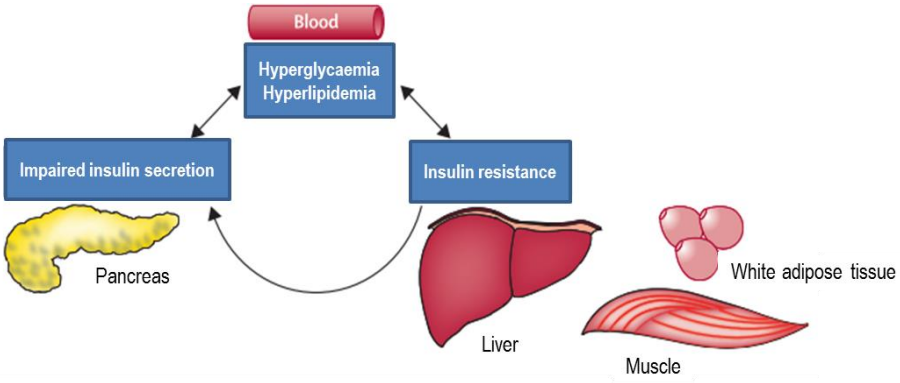


Figure 2. Type 2 diabetes (T2D) state. Figure adapted from Peirce & Vidal-Puig (2013) [14].

International Diabetes Federation calculates that the diabetic population worldwide will reach 645 million by 2040, which is equivalent to 1 in 10 adults [15]. World Health Organization (WHO) estimates that, globally, 422 million adults aged over 18 years were living with diabetes in 2014 (***table 1***) [16].

Table 1. Estimated prevalence of people with diabetes (18+ years). Table adapted from WHO (2016) [16].

WHO region	1980		2014	
	Millions	%	Millions	%
Africa	4	3.7	25	5.9
America	18	16.7	62	14.7
Eastern Mediterranean	6	5.6	43	10.2
Europe	33	30.6	64	15.2
South-East Asia	17	15.7	96	22.7
Western Pacific	29	26.8	131	31.0
Total	**108**	100	**422**	100

1.3. Relationship between obesity and type 2 diabetes

Several epidemiologic studies reveal a parallel increase of the twin epidemics of obesity and diabetes. Diabesity is a new term, which refers to diabetes occurring in the context of obesity. The increase in the prevalence of T2D is associated to the upsurge in obesity. It is estimated that about 90% of T2D is attributable to excess weight [17]. The connection between obesity and diabetes is associated to both, insulin resistance and insulin deficiency (***figure 3***).

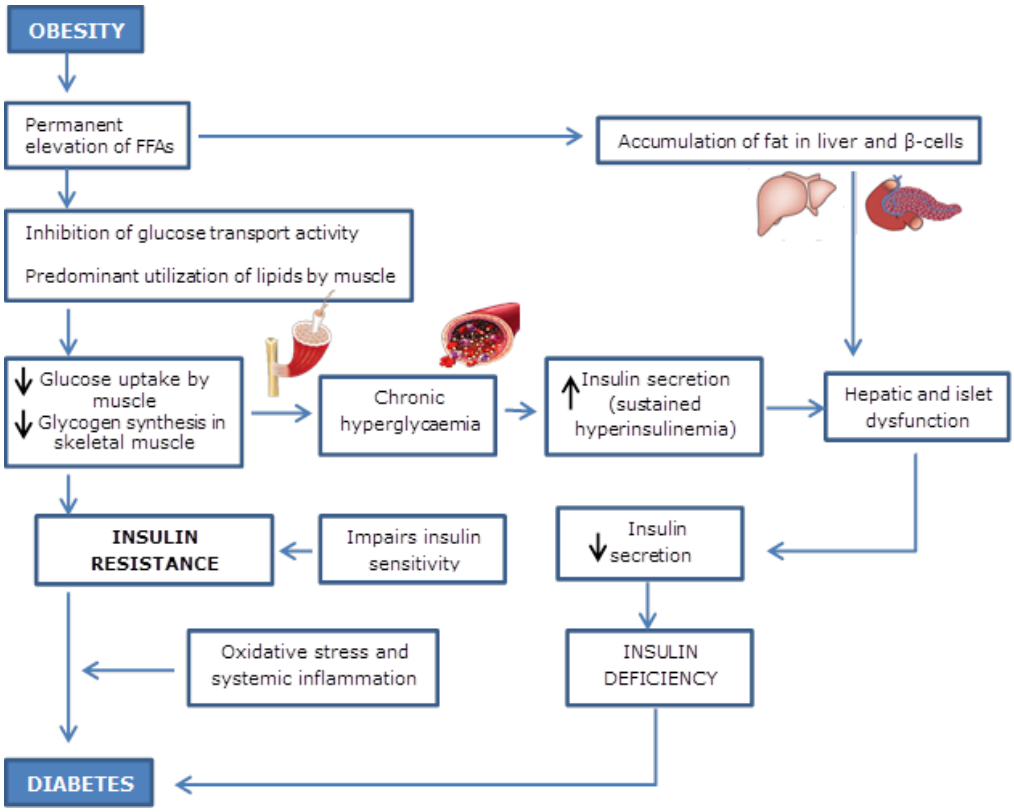


Figure 3. Links between obesity and diabetes. Figure adapted from Verma & Hussain (2017) [17].

Obesity causes sustained elevation of free fatty acids (FFAs) levels in plasma, both in the basal state and following glucose load, which present a major factor for insulin resistance. Increased plasma FFAs augment their cellular uptake and induce their mitochondrial β-oxidation, blocking at the level of substrate competition, intermediates accumulation, enzyme regulation, intracellular signalling and/or gene transcription of the glucose metabolism. The increase in lipid oxidation induces a diminution of glucose uptake by muscle and decreased rates of glycogen synthesis in skeletal muscle. This state of chronic hyperglycaemia further impairs insulin sensitivity.

Hyperglycaemia and compensatory hyperinsulinemia associated with insulin resistance and glucose intolerance lead to pathological glycation of circulating proteins. This progression ultimately leads to a pancreatic β-cell secretory failure and apoptosis (***figure 3***) [17].

Heritability for obesity and T2D is ~70 and ~35%, respectively [18,19]. Recent advancement in human genetics has led to the identification of a relatively big number of obesity- and T2D-associated genes, however, their contribution to the disease risk has been shown to be smaller than expected, greatly contributing other lifestyle factors [20].

2. Food components in obesity and type 2 diabetes

Foods and diet play a key role on both metabolic diseases. An unhealthy diet and some eating behaviours such as snacking/eating frequency, binge-eating patterns and eating out have been linked to obesity and finally to T2D [21]. In recent years, the Spanish Society of Community Nutrition (SENC) has been working on updating the 'Food Guidelines for the Spanish population' based on scientific evidence (***figure 4***)[22].

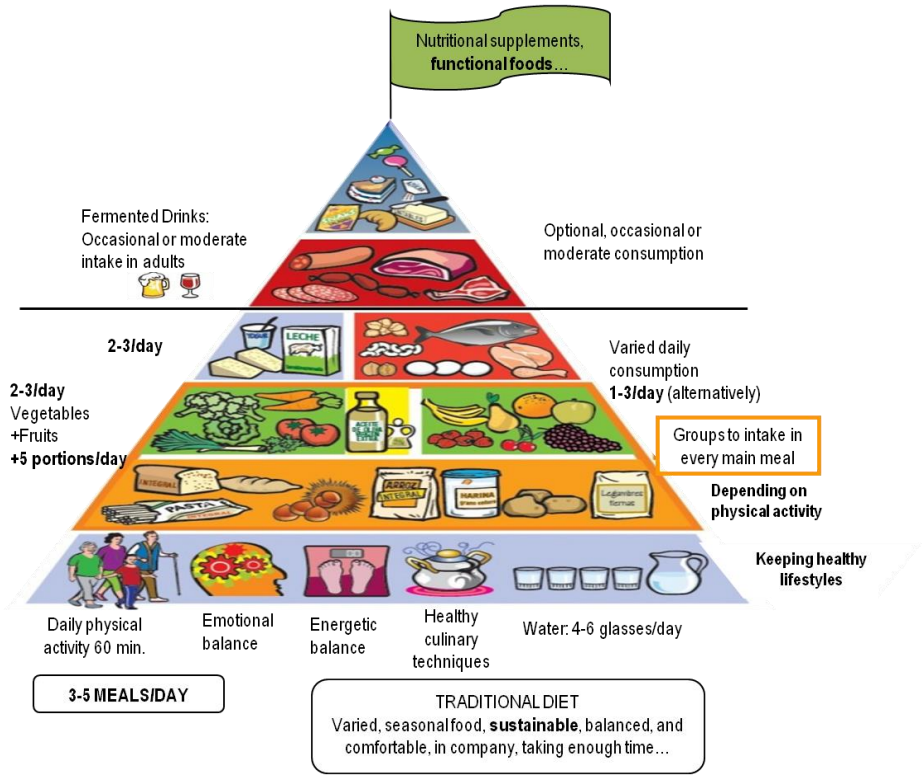
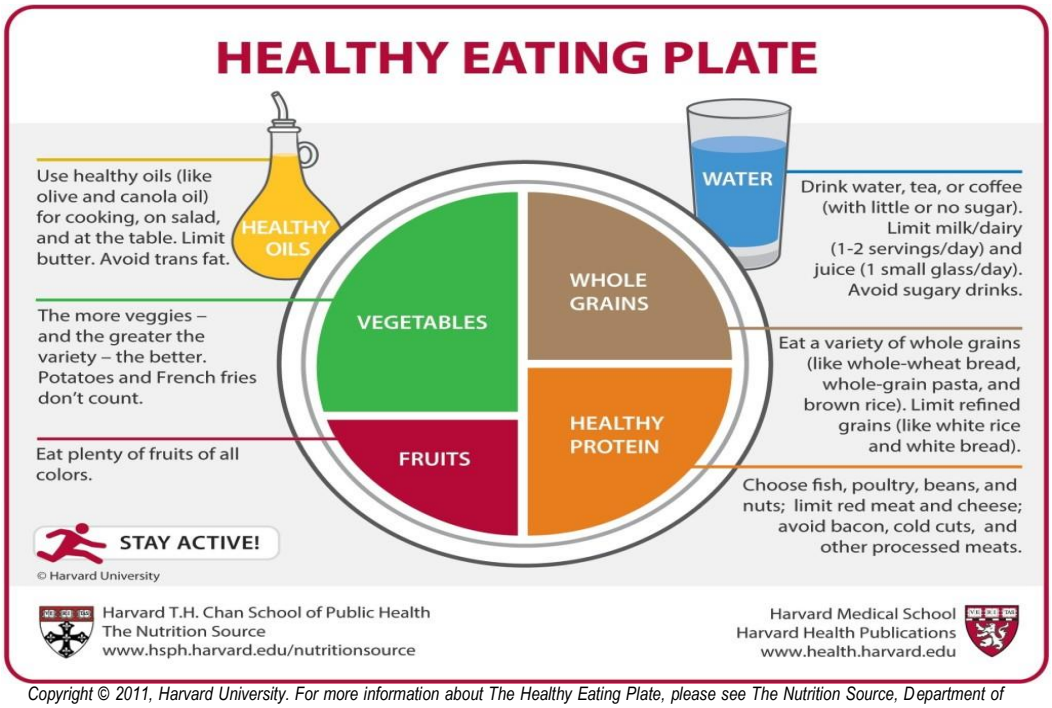


Figure 4. Healthy Eating Pyramid designed by the Spanish Society of Community Nutrition (SENC, 2015)[22].

Beyond the food recommendations themselves, the SENC has been paid special attention to healthy lifestyles advises which may affect greater than 40% on illnesses, i.e. physical activity and emotional balance. The intake of carbohydrates appears in its newer version as whole grains emphasizing the importance of fibre and the reduction of the frequent intake of sugary foods. On the top of the pyramid, a new tip appears regarding nutritional supplements, nutraceuticals and functional foods. Moreover, the SENC recommends healthy culinary techniques such as steaming, grilling and oven cooking, and a sustainable diet committed to the environment.

Furthermore, the Harvard School of Public Health pinpoints the importance of a quality diet. They propose to divide a plate in 4 parts: the half of the plate for vegetables and fruits, a quarter for whole grains and the other quarter for healthy proteins (***figure 5***).



Copyright © 2011, Harvard University. For more information about The Healthy Eating Plate, please see The Nutrition Source, Department of Nutrition, Harvard School of Public Health, www.thenutritionsource.org, and Harvard Health Publications, www.health.harvard.edu.

Figure 5. Healthy Eating Plate created by Harvard School of Public Health.

Previous studies support the protective role of diet, exercise and its combination in individuals genetically susceptible to both pathologies [20,23]. For example, insulin sensitivity and glucose uptake can be improved through regular physical activity and healthy diets that include sufficient fibre [16]. ***Table 2*** provides information on the role of lifestyle facts on the risk of obesity and T2D.

Table 2. Impact of lifestyle factors in obesity and type 2 diabetes (T2D). Table adapted from WHO (2003) [21].

Evidence	Increased Risk		Decreased Risk	
	Overweight/Obesity	**T2D**	**Overweight/Obesity**	**T2D**
Convincing	Sedentary lifestyles **High intake of energy-dense micronutrient-poor foods** [a]	Overweight and obesity Abdominal obesity Physical inactivity Maternal diabetes	Regular physical activity **High dietary intake of non-starch polysaccharides (dietary fibre)**	Voluntary weight loss in overweight and obese people Physical activity
Probable	Heavy marketing of energy-dense foods **High intake of sugars-sweetened soft drinks and fruit juices** Adverse socioeconomic conditions (in developed countries, especially for women)	Saturated fats Intrauterine growth retardation	Home and school environments that support healthy food choices for children Breastfeeding	**Non-starch polysaccharides**
Possible	Large portion sizes High proportion of food prepared outside the home (developed countries) ''Rigid restraint/periodic disinhibition'' eating patterns	Total fat intake Trans fatty acids	**Low glycaemic index foods**	n-3 fatty acids **Low glycaemic index foods**
Insufficient	Alcohol	Excess alcohol	Increased eating frequency	Vitamin E Chromium Magnesium Moderate alcohol

[a] Energy-dense and micronutrient-poor foods tend to be processed foods that are high in fat and/or sugars. Low energy-dense (or energy-dilute) foods, such as fruit, legumes, vegetables and whole grain cereals, are high in dietary fibre and water.

2.1. Nutrients

2.1.1. Digestible carbohydrates

Carbohydrates are an important part of the diet and their quality even yet. The structure will determine its digestibility, absorption and metabolism and therefore, the impact on the postprandial glucose and insulin concentration [24].

Jenkins et al. (1981) [25] pioneered the glycaemic index (GI) concept. It is a measure of the average concentration of glucose in the blood of subjects following a test food containing 50 g of available carbohydrate over 2 hours, and is expressed relative to glucose or white bread (GI = 100) [26]. Foods are categorized as low (≤55), moderate (56-69) and high (≥70) GI foods. Foods low on the GI scale tend to release glucose slowly and steadily, while foods high on the GI release glucose rapidly.

Food-related factors that influence the GI include: 1) the nature and amount of available carbohydrate (particle size, degree of hydration, nature of starch, etc.), 2) other food components (fat, protein, dietary fibres, phytochemicals); and 3) the food form, processing and preparation [26]. Research shows that similar amounts of carbohydrate from different foods can elicit different glycaemic responses (***figure 6***).

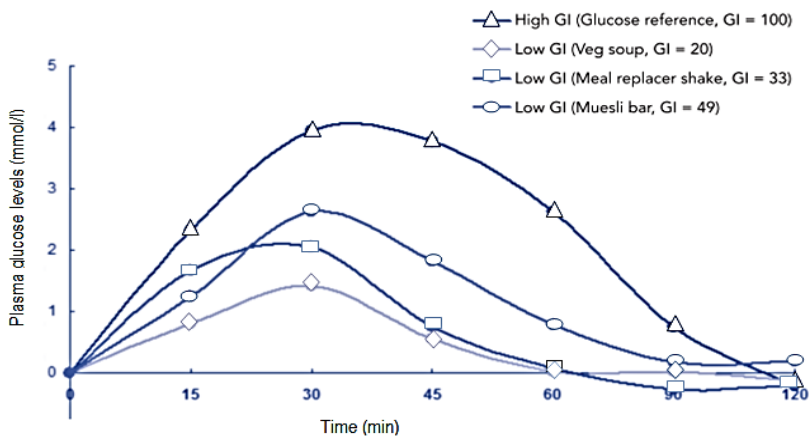


Figure 6. Blood glucose response curves for different foods (vegetarian food, meal replacer shake and muesli bar). Figure adapted from Sadler (2011) [26].

Moreover, the glycaemic load (GL) provides additional information about the glycaemic quantity in relation to a portion size (high (GL ≥ 20), medium (GL = 11-19) and low (GL = 0-10) GL foods). This concept incorporates the total available carbohydrate in the food portion. GL is the product obtained

by multiplication of GI and the amount of dietary carbohydrate. For an individual food, the GL is therefore more relevant than the GI. Some fruits and vegetables present high GI values, however their amount of carbohydrate is low (GL) and has little effect on blood glucose [24]. International tables of GI and GL values of foods have been updated [27]. ***Table 3*** presents a selection of the data correspond to common cereal-based foods and beverages**.**

Table 3. Glycaemic index (GI) and glycaemic load (GL) per serving, for selected common cereal-based foods and beverages, in subjects with normal glucose tolerance. Table adapted from Atkinson et al. (2008) [27].

FOOD	GI	Serving size (g)	GL per serving
BAKERY PRODUCTS AND BREADS			
Apple muffin, made with rolled oats and sugar	44±6	60	13
Apple muffin, made with rolled oats and without sugar	48±10	60	9
White wheat flour bread, average	75	30	11
Whole wheat bread, average	69	30	9
BREAKFAST CEREALS			
All-Bran®, average	44	30	9
Cornflakes®, average	81	30	20
BISCUITS			
Wheat based cereal biscuit	72±10	30	14
Wheat biscuits with extra wheat bran	61±4	30	10
SNACKS FOODS			
Corn chips, plain, salted	42	50	11
Potato chips, average	56	50	12
Pretzels, oven-baked	83±9	30	16
PASTA AND NOODLES			
Macaroni and Cheese (Kraft®)	64	180	33
Spaghetti, white, boiled, average	46	180	22
BEVERAGES			
Coca Cola® (US formula)	63	250 mL	16
Fanta®, orange soft drink	68±6	250 mL	23
JUICES			
Apple juice, unsweetened, average	41	250 mL	12
Pineapple juice, unsweetened	46	250 mL	16
SPORT DRINKS		250 mL	
Gatorade	78 ± 13	250 mL	12
Isostar	70 ± 15	250 mL	13

The term of “free sugars”, stablished by the WHO, includes monosaccharides and disaccharides added to foods and beverages, and sugars naturally present in honey, syrups, fruit juices and fruit juice concentrates [28]. There is increasing concern that intake of free sugars – particularly in the

form of sugar-sweetened beverages – increases overall energy intake and reduces foods more nutritionally adequate, leading to higher risk of these metabolic chronic diseases [29]. These beverages include soft drinks, fruit drinks, energy and vitamin water drinks. They are composed of naturally derived caloric sweeteners such as sucrose, high fructose corn syrup (HFCS), which is mostly composed of 45% glucose and 55% fructose, or even fruit juice concentrates [30]. A 20-year study on 120,000 men and women found that people who increased their sugary drink consumption by one 12-ounce serving per day gained more weight over time -on average, an extra pound every four years- than people who did not change their intake [31]. People who consume sugary drinks regularly, one to two cans a day or more, have a 26 % greater risk of developing T2D than people who rarely have such drinks [32]. The hydration pyramid proposed by the SENC recommends replacing the intake of sodas by water (mineral or tap water), tea and coffee in their natural forms (unsweetened) [22]. Likewise, these recommendations fit with those given by the Harvard School of Public Health in its Healthy Eating Plate.

Regarding the digestible complex carbohydrates, starch is classified into rapidly digestible starch (RDS) and slowly digestible starch (SDS)[33]. RDS is that digested within 20 minutes of enzymatic action and is common in fresh white bread, white rice, and freshly cooked potatoes. SDS is the starch fraction digested between 20 and 120 minutes and is mainly present in pasta, wholegrain bread, waxy maize starch, sorghum and legumes (***figure 7A***). RDS induces a fast increase in blood glucose and insulin levels and SDS causes slow and prolonged release of glucose into the blood stream, coupled to a low glycaemic response (***figure 7B***) [34]. Thereby, RDS can induce health complications (T2D) and SDS starch may be helpful in controlling and preventing hyperglycaemia-related diseases.

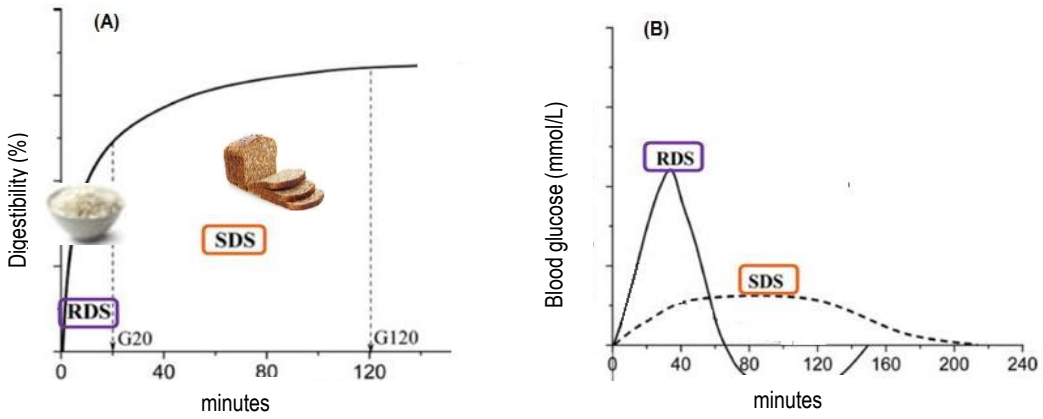


Figure 7. Nutritional starch fractions according to (A) their digestibility *in vitro* and (b) their *in vivo* glycaemic response. Rapidly digestible starch (RDS) and slowly digestible starch (SDS). Figure adapted from Miao et al. (2013) [34].

Energy-dense foods tend to be high in digestible sugars and starches. The fructose component in sugar causes directly dysregulation of lipid and carbohydrate metabolism, and, sugar indirectly promotes positive energy balance, thus body weight and fat gain, which also cause dysregulation of lipid and carbohydrate metabolism [35,36]. In fact, WHO strongly recommends reducing the intake of free sugars to less than 10% of total energy intake and suggests a further reduction below 5% of total energy intake to get additional benefits for health [28].

In this sense, the use of low-energy and low-GI sweeteners has gained increasing interest in the last years, due to the growing need as alternatives to sugar and to control the glycaemic responses [37]. The effects of low-energy sweeteners (LES) on energy intake and body weight have been the subject of many studies over the past 30 years. A very recent systematic review of relevant studies in animals and humans consuming LES with *ad libitum* access to food energy has been conducted [38]. A meta-analysis of short-term randomized controlled trials (129 comparisons) showed reduced total energy intake for LES versus sugar-sweetened food or beverage consumption with no difference with water. Meta-analysis of sustained intervention randomized controlled trials (4 weeks to 40 months) also presented that consumption of LES versus sugar led to relatively reduced body weight and a similar relative reduction in body weight versus water. Overall, the balance of evidence indicates that use of LES in place of sugar, in children and adults, leads to reduced energy intake and body weight.

Stevia is a natural source of non-nutritive sweeteners providing zero-calories that can be used as a substitute of sugar or artificial sweeteners. The main sweet components in leaves, approximately 250–300 times sweeter than sucrose, are stevioside and rebaudioside A (***figure 8***) [39].

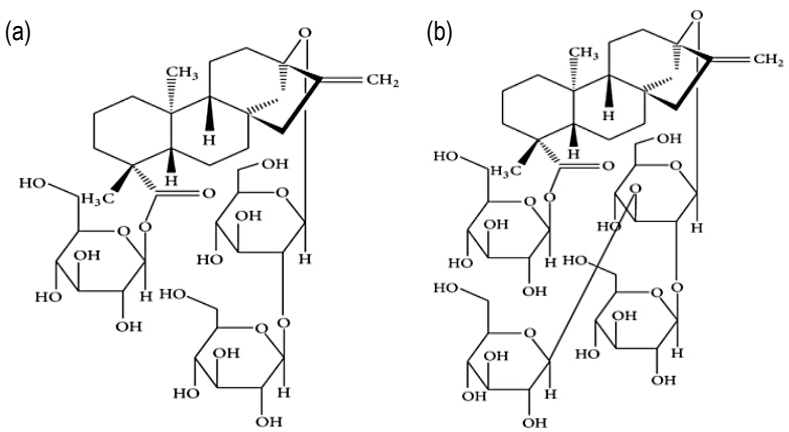


Figure 8. Chemical structure of the main sweet components in stevia leaves, (a) stevioside and (b) rebaudioside A.

The use of steviol glycosides (E-960) as food additive is accepted by the European Comssion (Regulation (EC) No 1333/2002 on food additives). A selection of foods, which can include in their formulation this non-nutritive sweetener, is defined in the Regulation (EU) No 1131/2011. Moreover, the European Food Safety Authority (EFSA) established an Acceptable Daily Intake (ADI) for steviol glycosides of 4 mg/kg body weight/day. Human digestive enzymes are not capable of hydrolysing the β-glycosidic bonds and the intestinal microflora converts steviol glycosides to steviol. Steviol glucuronide was found to be the main metabolite in plasma, which is eliminated via urine [40].

Stevia has been found to increase insulin sensitivity in rodent models [41] and to have beneficial effects on blood glucose and insulin levels in human studies [42,43]. Recent research has evaluated a possible effect of stevia on glucose transport and its molecular mechanism of action [39]. Results reveal that stevia extracts and insulin behave similarly, being stevia as efficient as insulin in increasing glucose uptake. The co-treatment with insulin and stevia extracts causes a rise of glucose transport significantly higher than the increase due to insulin alone. These findings suggest that the use of stevia extracts beyond their sweetening power and it may offer therapeutic benefits.

2.1.2. Non-digestible carbohydrates

Dietary fibre was traditionally classified as soluble and insoluble fibre and this division is still used in nutrition labelling, however, scientific evidence supporting that soluble fibres lower cholesterol and glucose, and insoluble fibres increase stool weight is inconsistent [44]. Resistant starch and inulin, both soluble fibres, do not appear to lower blood cholesterol and the insoluble fibre effect on stool weight is highly variable. Many fibre sources are soluble but still enlarge stool weight. Nowadays, EFSA defines dietary fibre as non-digestible carbohydrates plus lignin that comprises [45]:

- Non-starch polysaccharides (NSP): cellulose, hemicelluloses, pectins, hydrocolloids (gums, mucilage, beta-glucans);
- Resistant oligosaccharides: fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), other oligosaccharides that resist digestion (with three or more monomeric units);
- Resistant starch: physically enclosed starch, some types of raw starch granules, retrograded amylose, chemically and/or physically modified starches;
- Lignin naturally associated with dietary fibre polysaccharides.

Dietary fibre is a non-digestible nutrient that can be partially fermented in the colon by the microbiota. Short-chain fatty acids are released during its fermentation which are absorbed and available as metabolizable energy (2 kcal/g) [46]. The fibre is considered as a “nutrient of public

health concern" since low intakes are associated with potential health risks ((http://www.fda.gov/nutritioneducation). A daily intake of 25 g of total dietary fibre is set as a dietary reference value for a normal bowel function in adults, while consumption above 25 g of total dietary fibre per day may reduce risk of T2D and improve weight maintenance [45]. In this sense, a health claim regarding non-digestible carbohydrates such as FOS from inulin and their ability to reduce post-prandial glycaemic responses has been approved [47]. The fibre slows the breakdown of starch into glucose –thus maintaining a steady blood sugar rather than causing sharp spikes.

Dietary fibre is positively associated with enhanced weight control and obesity [48]. A higher intake of foods high in NSP contributes to a reduction in total energy intake and eventually in lower weight [21]. Resistant starch and inulin, which are sources of fermentable substrate for the large bowel microbiota may prevent body-weight and waist circumference gain [49]. Multitude of cross-sectional studies [50,51] and large observational studies [52,53] relate inversely dietary fibre intake and body weight gain. Regarding the possible mechanisms associated to effect of dietary fibre on weight reduction body and fat-mass regulation a complex of multiple factors is involved: central nervous circuits, peripheral sensation stimuli, mechanical and chemical satiation signals arising in the gastrointestinal tract, afferent vagal input, and adiposity signals from fat tissue and liver [54]. The potential biological mechanisms of the fibre associated with enhanced body weight regulation and weight loss are summarized in ***Table 4***.

Table 4. Potential mechanisms for fibre and fibre-rich diets on body weight regulation and weight loss. Table adapted from Dreher (2015) [55].

Target Level	Increase	Decrease
Food Intake	Food volume Chewing time	Diet energy density (fibre=2 kcal/g) Dietary fat intake Hunger
Stomach	Satiety signals (e.g., ghrelin)	Gastric emptying Lipid emulsification Hunger
Small intestine	Satiety signals (cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY)	Hunger
Circulation	Insulin sensitivity Short chain fatty acids Satiety signals	Postprandial glucose and insulin
Large intestine	Fibre fermentation Short chain fatty acids Prebiotic microbiota Unabsorbed macronutrients (fibre fat) and bile acids	Metabolizable energy
Body weight	Negative energy balance	Weight gain Total body fat gain Abdominal and visceral fat gain

On the other hand, dietary fibre has been associated with reduced risk of T2D. A 6-month randomized parallel study demonstrated an improvement in the daily blood glucose profile and glycated haemoglobin (HbA1C) levels with 50 g soluble fibre/day compared to 15 g/day [56]. In a study of more than 160,000 women whose health and dietary habits were followed for up to 18 years presented 30% less likely to develop T2D when consumed 2-3 servings of whole grains a day than those who rarely ate them [57]. Moreover, setting out a low GI diet results in an increased intake of unavailable carbohydrate (fibre) and in a concomitant decrease in GL. Moreover, lowering GL and raising unavailable carbohydrate both independently act to control fasting blood glucose similarly (***figure 9***) [58].

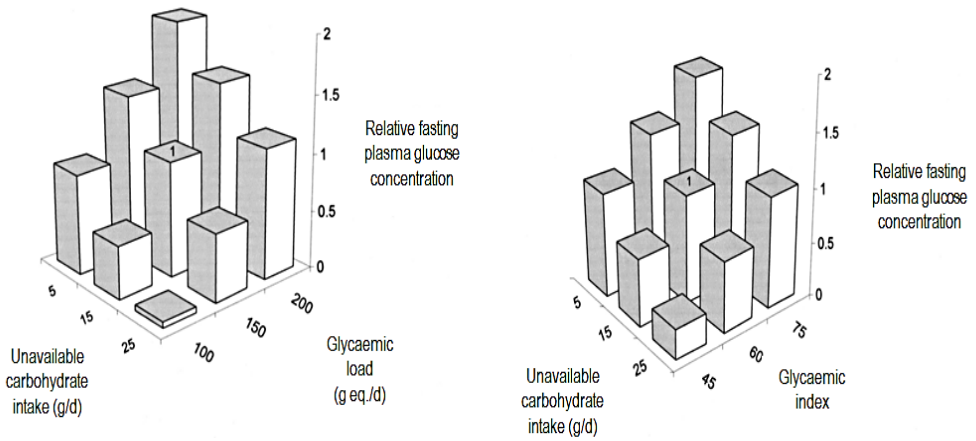


Figure 9. Implications of the effect of unavailable carbohydrate intake, glycaemic load (glucose equivalents/d) and glycaemic index on fasting blood glucose concentrations. Figure adapted from Livesey et al. (2008) [58].

Dietary fibre is mainly obtained from fruits, vegetables, cereals and legumes. Food processing of those products generates large amounts of by-products which contain significant fibre content [59]. The use of these natural sources of fibre is a sustainable approach in the management of chronic diseases such as obesity and T2D. Apple pomace is a by-product biomass that possesses high content of total dietary fibre (74%) and other functional properties such as density, water and oil holding capacity and swelling capacity including glucose dialysis retardation index (37%). Moreover, linked to this dietary fibre the phenolic compound, phlorizin, acts as an oral antidiabetic drug for the T2D by inhibiting sodium-glucose co-transporter-2 (SGLT 2) [60]. By-products from the coffee process such coffee pulp and husks are also sustainable source of dietary fibre, which involves phytochemicals adhered to its structure and might play and important role on reducing the risk of these chronic diseases [61].

2.2. Non-nutritive food components

2.2.1. Antioxidants

A variety of antioxidant compounds have been studied for their ability to exert anti-obesity and anti-diabetic effects [62,63] Their bioavailability is essential to achieve these health properties. The gastrointestinal tract may act as an extractor where both the mechanical and chemical action contribute to the extraction of these phytochemicals from the food matrix [64,65].

Coffee is a major source of antioxidants such as chlorogenic acid (CGA) (***figure 10***) [66]. Different mechanisms of actions have been proposed for the CGA to improve glucose and lipid metabolism: 1) activation of AMP activated protein kinase (AMPK), a master sensor and regulator of cellular energy balance which leads to increased glucose transport; 2) improvement of cellular mechanisms; 3) inhibition of the intestinal α-glucosidase activity; 4) alteration of glucose-dependent insulinotropic peptide (GIP) hormone and therefore insulin secretion [63].

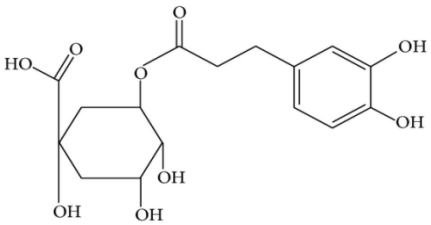


Figure 10. Chemical structure of chlorogenic acid.

Several studies have been reported anti-obesity effect of CGA [67,68]. CGA significantly lowers body weight, visceral fat, mass triglyceride and cholesterol concentrations in high-fat diet-induced-obese mice [67]. CGA presents anti-lipidemic effects in mice by inhibiting fatty acid synthesis in a dose- and time-dependent manner, enhancing dyslipidaemias and lipid profile [69]. Furthermore, CGA acts as a principle active in glucose metabolism regulation. A daily intake of 3 to 4 cups of decaffeinated coffee containing high CGA amounts significantly reduces the risk for T2D by 30% [70]. CGA is a novel insulin sensitizer that stimulates insulin secretion from rat islets of Langerhans [71] and improves glucose tolerance and insulin resistance in obese Zucker rats [72]. Very recently, our group has reported that coffee silverskin extract (CSE), which possess CGA, improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells [73] and demonstrated a chemo-protective effect in pancreatic tissue in rats [74], thereby reducing the risk of diabetes.

On the other hand, other dietary antioxidants may reduce reactive oxygen species (ROS) generated in T2D patients. These reactive species impair insulin signalling pathways and induce cytotoxicity in pancreatic β-cells. Neutralisation of oxidants by increased antioxidant availability may mitigate these effects. In a meta-analysis, significantly reduced HbA1C levels were observed by antioxidant supplementation, suggesting that antioxidants may have some benefit in protecting against the complications of T2D [75].

2.2.2. Alpha-glucosidase inhibitors

Alpha-glucosidase is a membrane bound enzyme located in the brush border of the enterocytes of the jejunum in the small intestine, which works to facilitate the absorption of digestible carbohydrates by catalysing the hydrolytic cleavage of oligosaccharides into absorbable monosaccharides [76]. By the inhibition of α-glucosidase in the intestine, the process of carbohydrate digestion spreads to the lower part of small intestine [77], therefore slowing the elevation of blood sugar following a carbohydrate meal. The mechanism of action of α-glucosidase in the absence and presence of α-glucosidase inhibitors is shown in ***figure 11***.

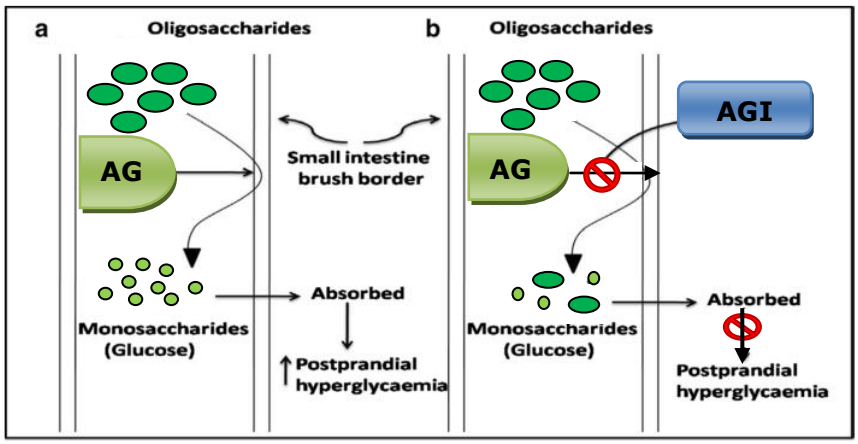


Figure 11. Mechanism of action of α-glucosidase on carbohydrates in the (a) absence and in the (b) presence of α-glucosidase inhibitors. [AG: α-glucosidase; AGI: α-glucosidase inhibitor]. Figure adapted from Patil et al. (2015) [78].

The main drawback of the current α-glucosidase inhibitor -acarbose- is its side effects such as abdominal distention, flatulence and diarrhoea [79]. Consequently, search of alternative natural compounds has rapidly increased. A wide range of phytochemicals present in plants and foods behave as α-glucosidases inhibitors so they are used as bioactive compounds for the glycaemic

control in T2D [80–83]. Normally, the bioaccessibility of the α-glucosidase inhibitors requires disruption of the cell walls and cellular compartments and cleavage from carbohydrates [84]. The majority of polyphenols appear to be released during the gastric phase by the pepsin action in conjunction with peristaltic movements and low pH, favouring the diffusion from the matrix [85,86].

CGA appears again as a potent α-glucosidase inhibitor, which has been described to act in a synergistic manner [63] or non-competitive manner in rats [87]. In a recent patent published by our group, it has been reported that CGA and CSE present inhibitor effect on the α-glucosidase enzyme *in vitro* [88]. Moreover, CGA inhibits α-glucosidase activity and reduces postprandial blood glucose in rats [89]. Other numerous coffee bioactive substances different to CGA present inhibitor effects on glucosidase enzyme, such as anthocyanin extracts from coffee pulp [90] or β-carboline alkaloid norharman [83]. Therefore, due to all these active principles present in coffee it may be possible a synergistic action against α-glucosidase activity.

On the other hand, different types of fibre present inhibition of these digestive enzymes. It might be attributed to several possible factors such as the presence of inhibitors incorporated on the fibre structure (i.e polyphenols) or even by direct adsorption of the enzyme on fibres, leading to decreased glucosidase activity [91]. However, further investigation would be required to gain insight into what types of sustainable fibres from food wastes, such as coffee fibres, may have inhibitor effect and their specific mechanisms of action.

Furthermore, aqueous extracts obtained from stevia leaves revealed to have effect on α-glucosidase by decreasing its activity that is retained after its incorporation on a food matrix [92].

2.2.3. Lipase inhibitors

Pancreatic lipase plays a key role in the efficient digestion of triglycerides. It removes fatty acids from the α and α' position of dietary triglycerides, yielding β-monoglycerides and long chain saturated and polyunsaturated fatty acids as the lipolytic products. Pancreatic lipase is responsible for the hydrolysis of 50–70% of total dietary fats [93]. In this context the inhibition of fat digestion is an interesting approach for reducing fat absorption and therefore body weight gain [94].

Orlistat, one of the two clinically approved drugs for obesity treatment, has been shown to act through inhibition of pancreatic lipase. However, this anti-lipidemic drug has certain unpleasant gastrointestinal side effects like oily stools, oily spotting, and flatulence among others. Therefore, research is required to identify newer pancreatic lipase inhibitors that lack some of these unpleasant

side effects [95]. Extracts from hundreds of species of medicinal plants, vegetables, and fruits as well as products from microorganisms, fungi and marine algae are being screened for potential lipase inhibitory activity [96].

It is well known that polyphenols from plants have an affinity for proteins, mainly through hydrophobic bonding. Many polyphenolic compounds such as flavones, flavonols, tannins and chalcones are active against pancreatic lipase [95]. Coffee that contains large amounts of phenolics acts as an anti-lipidemic agent by inhibiting pancreatic lipase. The major polyphenols, 5-caffeoylquinic acid (CQA), 4-CQA, and 3-CQA are stable and preserved in coffee after simulated digestion to inhibit the lipase [97]. Moreover, polyphenols other than CQAs, such as feruloyl quinic acid isomers or ferulic acid, exhibit a lipase inhibitory effect [98]. Taken together, the results suggest that the lipase inhibition activity of both CQA and dicaffeoylquinic acid (DCQA) drive the lowering effect of free fatty acid release during the simulated digestion in the instant coffee [97]. In addition, the CSE patented by our group has shown to exert inhibitory effect on the pancreatic lipase [99] as well as the CGA [88].

Dietary fibre can affect lipid metabolism through inhibition of pancreatic lipase. Wheat bran and wheat germ may inhibit pancreatic lipase and decrease intestinal absorption of dietary fats and cholesterol [100,101]. Citrus pectin strongly inhibits the activity of digestive lipases as well [102]. Moreover, it has been reported that dietary fibre becomes an important inhibitor of lipase when a basic group is introduced in its structure. Basic dietary fibres are strong inhibitors of gastrointestinal lipase reactions and are reversible reaction inhibitors [103].

Furthermore, stevia leaves is able to inhibit the activity of the pancreatic lipase, thereby acting as an inhibitor agent, useful for the management of obesity [104].

2.3. Food-processing compounds

The Maillard reaction (MR) is a series of reactions between proteins and carbohydrates that occur in foods during their storage and cooking, with the rate of reaction accelerating as temperature increases. The dietary MR products (MRPs) provide predominantly the characteristic colour and aroma of cooked foods. The main factors determining their rate of formation in food include nutrient composition (protein, carbohydrates and fat), temperature, duration of heat application, humidity, pH, and the presence of trace metals [105,106].

Briefly, MR starts by condensation of the carbonyl group of a reducing sugar with the amino groups of a protein generating Schiff bases, which rearrange to Amadori or Heyns products. These early MRPs undergo dehydration and rearrangements followed by other reactions, such as cyclisation, oxidation and dehydration, to form more stable advanced glycation end products (AGEs). Reactive dicarbonyl products such as methylglyoxal (MGO) are formed as intermediate products during all stages of the MR, but also as intermediates of glucose autoxidation and lipid peroxidation. The accumulation of these reactive products produces the formation of oxidative AGEs such as carboxymethyl-lysine (CML), and pentosidine [107]. ***Table 5*** presents the content of selected early MRPs and AGEs in different foods.

Table 5. Content of selected early MRPs and AGEs in different foods. Table adapted from Hellwig and Henle (2014) [108] and references therein, Uribarri et al. (2010) [105] and He et al. (2014) [109].

Food	AP (g/kg)	CML (mg/kg)	Pyrraline (mg/kg)	Pentosidine (mg/kg)	AGE (MU/kg)
Milk, pasteurized	0.1	0.2-0.5	n.d.	n.d.	0.01-0.1
Evaporated milk	7-18	46	0.4-3.2	0.02-0.04	-
Pasta	1-19	2.4-3.0	n.d-12	-	-
Bread	6-7	3-40	6-69	-	1.1-1.5
Bread crusts	-	37-94.3	60-240	0.03-0.18	0.4-0.7
Meat, roasted	-	2-20	-	-	47-100
Butter	-	0.3-0.4	-	-	233-265
Coffee, roasted	-	-	-	1.0-4.0	-
Biscuit, baked	-	50.7-117.5	-	-	13.4-14.7
Cookies, baked	-	-	-	-	0.9-32.2

AP: Amadori product expressed as fructoselysine; CML: Carboxymethyl-lysine; n.d.: not detectable; -: non-available data

Melanoidins are high molecular weight and brown-coloured compounds generated in the late stages of the MR. These products are mainly responsible of the specific colour and typical appearance of processed foods [110]. Due to their complex structure melanoidins can be defined as a type of “maillardized fibre”. It is very complex to structurally separate fibre from melanoidins and vice versa because carbohydrates are integral components of coffee melanoidins [111]. The current importance of melanoidins is linked with their health-promoting properties. Many beneficial effects have been associated to these compounds, such as antioxidant, antimicrobial, anti-inflammatory, antihypertensive or prebiotic activity [112]. On the other hand, acrylamide and 5-hydroxymethylfurfural (HMF) are mainly formed through MR and can be regarded as the most important heat-induced neo-contaminants occurring in bakery products. Many studies indicate that acrylamide is neurotoxic in animals and humans and that it is a reproductive toxicant, germ-cell

mutagen and carcinogen in rodents [113–115]. HMF has been shown to be converted *in vivo* to a 5-sulfoxymethyfurfural which is a genotoxic compound [116].

Dietary AGEs are partially absorbed (10–30%) [117,118] and a rapid rising peak of AGEs in serum is observed after consumption of a meal [119]. These dietary AGEs together with those endogenously produced have important implications in health. AGEs have been largely associated to oxidative stress and inflammation, eventually causing most chronic diseases such as T2D and obesity [120,121]. A diet low in AGEs seems to improve hyperinsulinemia (~40%) in fully treated T2D patients, confirming that exogenous AGEs actively participate in the metabolic dysfunctional milieu of T2D [122]. Moreover, dietary AGEs have been related to the development of insulin resistance and diabetes in animals, and more recently also in humans [123]. New studies have introduced an instructive view proposing that the abundance of pro-oxidant AGEs could potentially account for the initiation and progression of pre-diabetes to diabetes in the highly industrialized modern food environment [118]. On the other hand, certain dietary MRPs are largely undigested by human gut enzymes and are degraded by the colonic microbiota, mainly those in the early staged of the MR [124]. Some of these MRPs can play a role in colon toxicology, through increased colonic protein fermentation, and may also act as systemic toxicants and inducers of inflammation [125]. They may adversely alter the colonic microbial composition, potentially enhancing their risk for the development of metabolic diseases such as obesity and T2D [126]. More research needs to be carried out in this sense.

3. Satiety and chronic diseases

Satiety, the feeling of fullness that persists after eating, is an important factor in suppressing overconsumption, which can lead to overweight and obesity [127]. The literature demonstrates that foods that target within-meal satiation and post-meal satiety provide a plausible approach to weight management [128]. Foods that generate strong satiety sensations have obvious benefits for these chronic diseases.

3.1. "Foods for Specific Groups (FSGs)" and "Functional Foods"

A new regulation commonly referred to as the Food for Specific Groups Regulation or FSGs (Regulation (EU) No 609/2013) has replaced the Directive 2009/39/EC on foodstuffs intended for particular nutritional uses (PARNUTS) from 20th July 2016. The FSGs regulation abolishes the concept of dietetic foods and provides limited number of well-established and defined categories of

food that are considered as essential for certain vulnerable groups of the population: "processed cereal based food"; "food for special medical purposes"; "infant formula and follow-on formula"; and "total diet replacement for weight control" are the four categories for FSG. Diabetic food group is, however, under the Regulation (EC) No 1924/2006 on nutrition and health claims.

Regarding the commonly known functional foods, the Functional Food Center (FFC) has recently proposed a new definition: "*natural or processed foods that contains known or unknown biologically-active compounds; the foods, in defined, effective, and non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease"*. This definition pay attention to the bioactive compounds, such as phenolic acids, fibres, and polysaccharides [129].

Nutritional and health claims, which are under the European Regulation (EC) No 1924/2006, are used to legally highlight particular beneficial effects of functional foods with scientific evidence enough to declare on the product label or in advertising:

- Nutrition claim states, suggests or implies that a food has particular beneficial nutritional properties due to: 1) the energy it provides at a reduced or increased rate or does not provide; and 2) the nutrients or compounds it contains, contains in reduced or increased proportions or does not contain. "Low sugar", "with no added sugars" or "high fibre" are some of the permitted nutrition claims which may be closely related to low-energy dense and low-GI foods.
- Health claim states a relationship between food and health: 1) "Function Health Claims" relating to the growth, development and functions of the body, or psychological and behavioural functions, or slimming/ weight-control; 2) "Risk Reduction Claims"; and 3) "Claims referring to children's development". Some of the multitude proposed "Function Health Claims" that would be associated to those functional foods exerting enhanced satiety are "helps you to feel full for longer", "helps to manage body weight" or "promotes satiety".

The development and design of functional foods or foods for special medical purposes have a key role to play in combating certain chronic diseases such as obesity and T2D [130]. Enhanced satiety foods directly promote reduced food intake by lessening the effect of sensations of hunger [131].

3.2. Physiological mechanisms of satiety

The sensation of satiety is dependent on more than just the metabolic effects of nutrients in the gut and intestine [132]. The satiety cascade model proposes that even before food arrives in the gut,

cognitive and sensory signals generated will influence not only how much is eaten at that eating episode (satiation) but also in the period after consumption (satiety). These early satiety signals will integrate with post-ingestive and post-absorptive signals to determine satiety.

A schematic figure of the pathways involved in communication of satiation and satiety between the body and the brain is presented in ***figure 12.*** When the food is ingested, the gastric distension is communicated to the brain via the vagus nerve, initiating satiation. Gut hormones released from the stomach and intestine as a consequence of the food components reaction send signals to the brain, acting on that areas of the brain involved in appetite. Moreover, leptin from adipose tissue and insulin from the pancreas act on the brain to modulate satiation and satiety in the longer term. All these signals are integrated in the brain to affect energy intake.

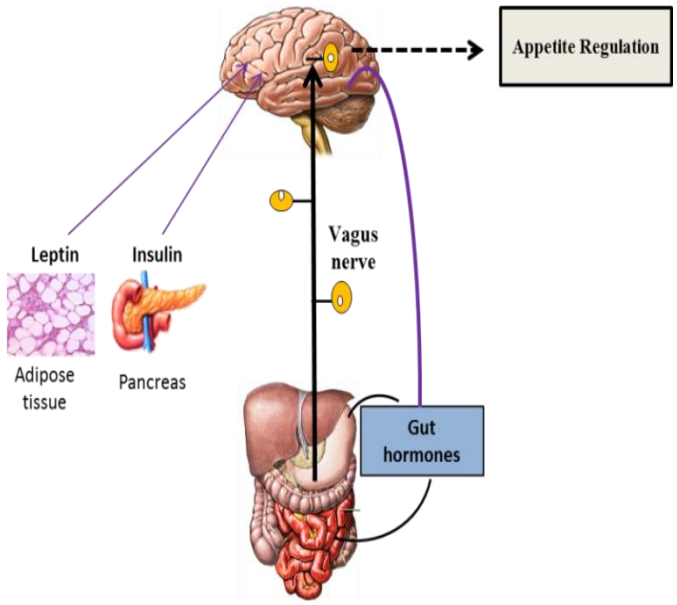


Figure 12. Schematic pathways for the physiological satiation and satiety signalling mechanism. Adapted from Benelam (2009) [127].

Broadly, satiety is influenced by both, short term ("episodic" signals in response to the consumption of food) and longer term ("tonic" signals indicating the levels of energy stores in the body). Focusing the attention on the short term, a number of hormones are secreted from the gut to indicate that food has been consumed. Ghrelin, cholecystokinin (CCK), oxyntomodulin (OXM), glucagon-like peptide-1 (GLP-1), peptide YY (3-36) (PYY 3-36) and pancreatic polypeptide (PP) are some of the gut-derived hormones involved in satiety.

GLP-1 and serotonin can be secreted in response to chemical and mechanical stimuli during meals, [133,134]. Both are mainly synthesized by the enteroendocrine cells of the gastrointestinal tract [135,136], causing an enhance of the feeling of fullness and therefore regulating the appetite. In addition, GLP-1 decreases blood glucose levels during hyperglycaemia by stimulating insulin secretion and reducing glucose-dependent glucagon secretion [137]. Very recently, a mechanism for intestinal secretion of these gut-derived hormones has been proposed (***figure 13***) [138]. Dietary compounds may stimulate GLP-1 release from enteroendocrin cells (EEC) via interaction with a G-coupled protein receptor (GPCR) (***figure 13A***), simultaneously the nutrient may stimulate extra-intestinal serotonin concentrations from enterochromaffin cells (ECC) via interaction with a GPCR (***figure 13B***). Consequently this induced serotonin release may stimulate (additional) GLP-1 release via interaction with a 5-HT receptor (5-HTR) on EEC (***figure 13C***).

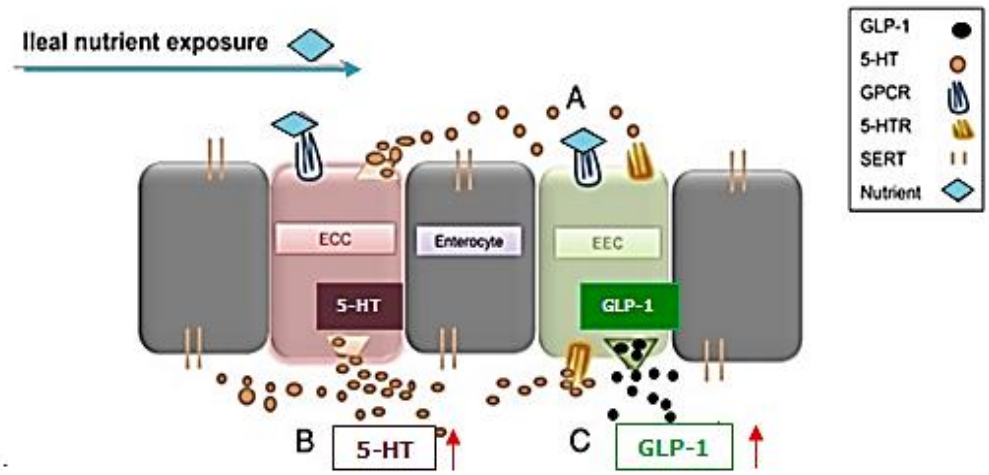


Figure 13. Proposed mechanisms of secretion for serotonin (5-HT) and glucagon-like peptide-1 (GLP-1) by modulation of dietary compounds in the ileum. Figure adapted from Ripken et al. (2016) [138].

3.3. Satiating food components

3.3.1. Nutrients

Proteins

Protein generally increases satiety and in this way it may facilitate a reduction of food intake [139–141]. Research suggests that the type of protein matters in inducing satiety and they should not be considered generically [139]. Dietary intake of proteins may be effective in satiety due to their ability

to increase GLP-1 secretion [137]. After ingestion, proteins are partially hydrolysed by pepsin in the stomach, and the digestion is completed in the small intestine by pancreatic proteases. The resulting peptides are able to stimulate GLP-1 release by the intestinal enterocytes [142]. Digested wheat proteins exert strong effects on the satiety and anti-diabetic effect through GLP-1 stimulation [143,144].

Carbohydrates

The effect of carbohydrates on satiety depends on the form of the carbohydrate and other aspects of the food such the dietary fibre content from which it is delivered. Regarding the non-digestible carbohydrates, different mechanisms have been proposed for the satiating effect of the fibre: 1) Higher mastification, which increases satiety signals to the brain; 2) Gastric distension due to the ability of soluble fibres to form gels and thereby signal sending to the central nervous system; 3) Belated nutrient absorption, as for instance, lower glucose- and insulin responses, and thereby delayed hunger and reduced energy intake at the next meal; and 4) Gut hormones release (i.e. GLP-1) as a consequence of the slower absorption of macronutrients in the first part of the small intestine prolonging their contact in the last part of the small intestine [145]. Moreover, several factors appear as main contributors of the satiating effect of the fibre: amount, extent to which the food was refined or processed, particle size in grain-based foods and energy density of fibre are important [146].

The literature on dietary fibre and satiety has been reviewed by several authors [146,147]. The intake of 16 g of FOS per day leads to higher feeling of satiety and less energy intake [148]. The mechanisms could be related to changes in the levels of blood hormones that have an effect on satiety. It was found with rodent trials that blood levels of GLP-1 and ghrelin changed in such a way that appetite was suppressed [149,150]. In a longer study (120 days), 55 overweight women took a daily supplementation of 0.14 g/kg of FOS and satiety sensation was greater with a significant decrease in body weight [151]. More recently, a positive effect on satiety and hunger was found in normal volunteers with the intake of coffee enriched with inulin and dextrin [152]. Furthermore, galactomannan may enhance satiety by forming a viscous gel in the stomach, which slows gastric, thereby enhancing fullness. In a clinical trial, an 8-gram dose significantly increased mean ratings of satiety and fullness in comparison to 0 or 4 grams of galactomannan fibre [153].

3.3.2. Non-nutritive food components

Food can contain a variety of bioactive compounds that may increase satiety and decrease energy intake. The coffee beverage has shown to enhance intestinal serotonin [154] and GLP-1 tested on

cells, mice [155] and humans [156]. Bioactive compounds present in the coffee drink such as CGA and caffeine, and non-nutritive sweeteners such as stevia are some of the satiating compounds.

Stevia

Stevia sweetener, in particular rebaudioside A, is able to induce satiety hormone release such as GLP-1 and peptide YY, at various locations of the small intestine using porcine *ex vivo* model [137, 153]. These findings may contribute to improve weight management and glucose control. However, clinical studies are necessary to confirm the potential satiety-inducing properties of rebaudioside A.

CGA

The main phenolic compound of the coffee beverage, CGA seems to enhance the satiety via stimulation GLP-1 hormone [71,157]. High increase of this hormone secretion is observed in intestinal secretin tumour cell line (STC-1) [157]. Additionally, the intake of 12 g decaffeinated coffee, 1 g chlorogenic acids and 500 mg trigonellines increases GLP-1 appear 30 min after compared to the placebo group [158].

Caffeine

This bioactive compound present in coffee has been greatly related to appetite control. The potential effects of different doses of caffeinated coffee (low, 3 mg/kg body weight and high, 6 mg/kg body weight) on appetite feelings and energy intake of male and female healthy volunteers were observed [159]. Coffee containing 3 mg caffeine/kg body weight induced a significant reduction in hunger feelings at 15, 30 and 60 min compared to water, and the consumption of high-caffeine coffee led to a lower energy intake at the *ad libitum* meal. A randomized placebo-controlled, double-blind study of overweight people showed that high-caffeine (524 mg/day) increases satiety compared with a low-caffeine diet (151 mg/day) [160].

In view of the widespread consumption and availability of coffee, and its content in bioactive compounds, the use of coffee-derived products may be very useful in the design of functional foods with potential properties for the preventative tool for obesity and T2D. These bioactive compounds, CGA and caffeine, and dietary fibre, are found at active physiological concentrations in coffee wastes. Therefore, this supports the use of coffee by-products as natural sustainable sources of bioactive compounds in the management of metabolic chronic diseases.

4. Coffee silverskin and spent grounds as natural sustainable sources of functional ingredients for chronic diseases

Coffee is mainly commercialized as a beverage obtained from ground roasted beans. During this processing, over 90% of the coffee cherry is discarded as an agricultural waste, which contains health promoting phytochemicals and nutrients [161]. Due to the great demand for coffee, large amounts of wastes are generated in the coffee industry. The interest in valorisation of agronomical by-products into diverse and useful novel products to achieve a global sustainable world has been recently published [162]. ***Figure 14*** represents coffee silverskin (CS) and spent coffee grounds (SCG) production employing the wet process of the bean and the instant coffee processing, which are studied in the present investigation.

CS represents 4% (w/w) of the coffee cherry. It is a thin tegument of the outer layer of the two beans forming the green coffee seed obtained as a by-product of the roasting process. SCG are the most abundant coffee by-product (45%) generated during the treatment of coffee powder with hot water to prepare coffee infusion or steam for the instant coffee preparation [163]. About 2 kg of wet SCG are obtained from each kg of instant coffee produced, with an annual generation of around 6 million tons worldwide [164].

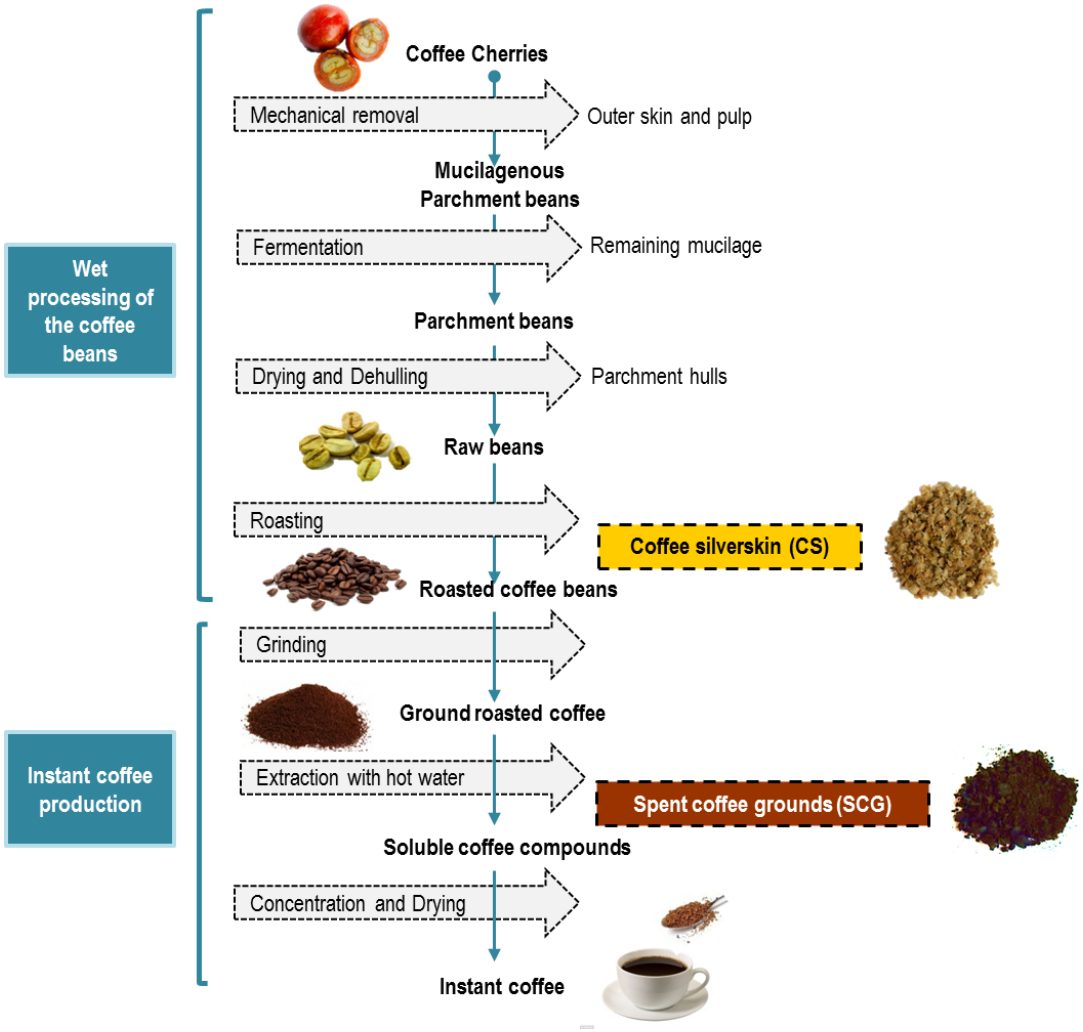


Figure 14. Diagram of coffee silverskin and spent coffee grounds production from the wet processing of the bean and the instant coffee process. Figure adapted from Fernandez-Gomez et al. (2016) [165].

CS has a high dietary fibre content (68-80%), which includes about 85% insoluble dietary fibre and 15% soluble dietary fibre [166,167]. Polysaccharides are also abundant components (60-70%) in CS [166,168–170] and sugars are polymerized into cellulose and hemicellulose structures which contain glucose, xylose, galactose, mannose and arabinose. CS contains protein, fat, and ash, at 16–19%, 2–3%, and 5-7%, respectively [61]. This coffee by-product presents phenolic compounds, mainly CGA, and other phytochemicals that contribute to its high antioxidant capacity. Moreover, it has been proposed as a natural source of prebiotic carbohydrates and dietary fibre [166,171].

Our research group patented an aqueous CSE from Arabica (*Coffea arabica*) and Robusta (*Coffea canephora*) (WO 2013004873 A1) enriched in caffeine and CGA and obtained using an environmentally friendly technology [99]. The chemical composition of the patented CSEs is summarized in ***table 6***. CSEs present a total dietary fibre content ranging from 29 to 36%, including 4-9% insoluble dietary fibre and 24-26% soluble dietary fibre. CSEs are a good source of polyphenols, in particular CGA (1-7 %), being the most relevant 5-O-, 3-O- and 4-O-caffeoylquinic acids [172]. CSE is also a good source of caffeine (3 %) and melanoidins (17-23 %). Melanoidins are formed during coffee roasting process [172]. As we previously mentioned melanoidns form part of the Maillardized fibre and contribute to the antioxidant capacity of the coffee and coffee wastes [173].

Table 6. Chemical composition of coffee silverskin extracts (adapted from del Castillo et al. (2013), Mesías et al. (2014) [99,172]) and spent coffee grounds (adapted from del Castillo et al. (in press)[61]).

Compounds	ACSE	RCSE	SCG
Fat (%)	-	-	1.6-2.3 [169,170]
Proteins (%)	5.36 [99]	0.99 [99]	13-17 [169,170,174]
Carbohydrates (%)	5.44 [99]	13.43 [99]	71-75 [170,174]
Total dietary fibre (%)	28.69 [172]	36.21 [172]	54-60 [169,170]
Soluble dietary fibre (%)	24.01 [172]	26.80 [172]	6-16 [169,170]
Insoluble dietary fibre (%)	4.67 [172]	9.41 [172]	47-50 [169,170]
Caffeine (%)	3.02 [99]	3.39 [99]	0.2-0.8 [175–177]
Melanoidins (%)	17.26 [172]	23.94 [172]	13-25 [170]
CGAs (%)	1.12 [172]	6.85 [172]	0.3-1.4 [170,175,176]
Total phenolic content (%)	3.10 [172]	3.54 [172]	1-17 [170,175,176]
ORAC (µmol trolox/g)	1194 [172]	1513 [172]	1821-2594 [175]
DPPH (µmol trolox/g)	219.9 [172]	231.3 [172]	74-112.1 [176]
ABTS (µmol trolox/g)	85.20 [172]	225.8 [172]	167.24-493 [170,176]
FRAP (µmol trolox/g)	829.8 [172]	640.1 [172]	498 [170]

Values are expressed on the dry matter basis. Superscript numbers correspond to the cited literature. ABTS, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; ACSE, Arabica coffee silverskin extract; CGAs, chlorogenic acids; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, Ferric reducing antioxidant power; ORAC, Oxygen radical absorbance capacity; RCSE, Robusta coffee silverskin extract; SCG, spent coffee grounds.

Regarding the chemical composition of SCG, polysaccharides are the major macronutrients (75%) (**table 6**). Among them, dietary fibre is the most important fraction (43-54%), being the insoluble fibre predominant (47-50%). SCG also contain protein, fat and ashes (14-17%, 2.3% and 1.3-1.6%, respectively). The polysaccharides are mainly constituted by 37% mannose, 32% galactose, 24% glucose and 7% arabinose. Caffeine content ranges from 0.2 to 0.8% depending on the caffeine extraction process and SCG variety. Different health-related chemicals bound to dietary fibre and

proteins such as phenolic compounds, mainly CGAs, have been reported in SCG from different sources [170,176]. The amount of coffee melanoidins that remained in SCG was approximately 15%. Coffee melanoidins fractions are diverse and possess different potential health benefits [173].

The feasible applications of these coffee by-products have been mainly focused in the production of biofuels, composts, animal feed and specific materials such as biosorbents, enzymes, chemicals and cosmetics among others [61]. However, due to the highlighted chemical composition of these coffee by-products and the amount of bioactive compounds present in them. Recently, great interest has been taken for the development of novel value-added applications related to food and health, derivatives from the biorefinery concept in the coffee sector [163,169,178]. Nowadays, some of these novel applications just begun, while others have started to be patented and only a few are being industrially used by the moment [61,161,179].

CS due to its bioactive compounds has been associated to food technology and health properties. This coffee by-product can be used as prebiotic carbohydrate [166]. CS is involved in the production of FOS due to its great potential to immobilize cells as well as to serve as nutrient for the microorganisms [180]. Short chains of FOS are produced, which have more prebiotic activity and stronger sweetness [181]. Moreover, CS is used to obtain innovative coffee blends rich in bioactive compounds [182] and it has been also employed in the formulation of bread to reduce caloric density and increase the dietary fibre content [168]. On the other hand, our research group has recently reported new evidence on CSE and its effects on reducing the risk of metabolic chronic diseases. This is the first time it has been described a chemo-protective effect of the patented CSE in pancreatic tissue in Wistar rats. The metabolism of CGA and caffeine, which are present in the CSE, starts in the gastrointestinal tract during digestion and thereby their metabolites are able to protect the pancreatic cells against the risk of diabetes [74]. Moreover, new data has been reported by our group about the capacity of this CSE to improve glucose-stimulated insulin secretion and to protect against streptozotocin-induced damage in pancreatic INS-1E beta cells [73]. New evidence shows that the patented CSE inhibits AGEs formation by different pathways including protein-phenol conjugation. CGA seems to be a principal contributor to the antiglycoxidative properties of the CSE [183]. Therefore, taking into account all these new findings provided by our group, CSE may be used as a nutritional supplement or food ingredient to reduce the risk of metabolic syndrome diseases such as T2D.

Regarding the SCG, they have been used as additives and coadjuvants. Beta-glucosidase is covalently immobilized onto SCG for the conversion of isoflavone glycosides into their aglycones in black soymilk, which presents higher biological activities than their respective glycosides [184]. A

distilled beverage with a coffee aroma has been produced from SCG [185] [185]. In addition, dietary fibre can be extracted from SCG employing different processes such as ohmic technology [186,187], alkaline hydrogen peroxide treatment [188] and autohydrolysis [189]. This dietary fibre is fermented by colon microbiota producing metabolites that exhibit strong anti-inflammatory potential by suppressing nitric oxide production and inhibiting inflammatory mediators such as IL-10, CCL-17, CXCL9, IL-1β and IL-5 cytokines. As a consequence, SCG have been proposed as protective agent against the onset and/or progression of chronic inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis. SCG have been also proposed as antimicrobial agents, which might be related to the melanoidin content [170]. However, to the best of our knowledge, there are not previous studies reporting the use of SCG as food ingredient and their effects on the energetic metabolism and satiety.

Therefore, the use of these coffee by-products with potential to be valorised into food ingredients with health promoting properties related to the metabolic chronic diseases, will contribute to a sustainable nutrition for the population.

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Hypothesis, objectives and work plan

HYPOTHESIS, OBJECTIVES AND WORK PLAN

Obesity and T2D have reached epidemic proportions in the past few years becoming serious health concerns. Both are related multifactorial complex diseases, and a large proportion of the cases are preventable. Nowadays, food choice, toxic food ingredients, nutritional deficiencies and lack of physical exercise cause more than 95% of all chronic diseases. In the last decades, the market of functional foods has surprisingly increased. Many plant extracts are emerging as functional candidates for the reduction of risk of non-communicable chronic diseases. Plant food by-products are sustainable sources of bioactive compounds with health promoting and therapeutic properties to satisfy consumer's demands.

Coffee is one of the most frequently consumed drinks in the world. Its total production in 2014-15 was of 142 million 60 kg bags (International Coffee Organization, 2015). Several epidemiological studies document the protective effect of coffee components in the risk of chronic diseases such metabolic disorders associated to oxidative stress and inflammation including diabetes such dietary fibre and CGA among others bioactive compounds.

CS is the only by-product produced during the roasting process in large amounts worldwide. SCG are the residual material obtained during the preparation of the coffee beverage or the instant coffee. On average, one ton of green coffee generates about 650 kg of SCG, and about 2 kg of wet SCG are obtained for each kg of soluble coffee produced. CS and SCG also contain appreciable amounts of bioactive nutrients and non-nutritive compounds. Coffee by-products are very abundant worldwide and they contain several health promoting compounds able to improve the metabolism of carbohydrates and lipids and to reduce the risk of chronic diseases. Coffee industry needs to recycle large amounts of wastes in order to reduce its environmental impact. **In conclusion, the valorisation of coffee wastes using the bio-refinery approach represents a great opportunity for the sustainability and competitiveness of the coffee sector and the industry of functional beverages and foods for chronic diseases.**

The health effects of CS and SCG as ingredients of foods and beverages largely will depend on the bioaccesibility and bioavailability of their bioactive components in the organism. Digestion has a great impact on these events. Components released during this physiological process may affect satiety, bioactivity of food components and food safety. Few information is available on how the interaction between the digestion products of nutrients and non- nutrients affect health and it is of great interest.

The **general objectives** proposed for demonstrating the hypothesis of the present research were as follows:

1. To validate the use of coffee wastes as sustainable food ingredients.
2. To provide new knowledge on interest of the conversion of coffee by-products, CS and SCG, in functional ingredients to be employed in the elaboration of sustainable beverages and foods for chronic metabolic diseases presenting high sensorial quality.
3. To study the impact of the digestion process of functional foods for chronic diseases containing coffee by-products on the release of satiety hormones and formation of compounds of interest in health and diseases (advanced glycation products).

The **specific objectives** of the present thesis were as follows:

1. **To validate the utility of CS and SCG as food ingredients** *(Chapters 1 and 2)*
   To achieve this goal, novel beverages based on CSE (***Chapter 1***) and bakery products based on CS or SCG (***Chapter 2***) were developed. The physical, nutritional and sensorial quality of the novel products was evaluated. Safety regarding processing neo-contaminants with health impact, and bioaccesibility *in vitro* of the bioactive components were examined on CS- and SCG-containing biscuits. Microbiological quality of SCG-containing biscuits was assessed.

2. **To determine the biological effects of CSE and SCG as food ingredients to reduce the risk of obesity and T2D** *(Chapters 1 and 2)*
   To achieve this goal, it was evaluated the effects of novel beverages based on CSE on body fat reduction *in vitro* and *in vivo* employing as animal model *Caenorhabditis. elegans (C.elegans)* (**Chapter 1**). Moreover, *in vitro* effects of the bioaccessible food components released during the simulated human digestion of SCG-containing biscuits on α-glucosidase activity and satiety hormones were examined (**Chapter 2**). The satiating effect was evaluated in gut intestinal cells by the measurement of GLP-1 and serotonin release.

3. **To evaluate the digestion process on the release of compounds with health impact** *(Chapter 3)*
   To achieve this goal, *in vitro* oral gastrointestinal digestion of simplified meal systems comprising an average meal and high-sugar meals was performed. The formation of MRPs associated to the pathogenesis of diabetes and its complications were examined.

Figure 1 shows the work plan performed to achieve each objective proposed in the thesis.

NOVEL BEVERAGES

CSE

Evaluation of beverage quality

Beverages (ACSE and RCSE)
Nutritional analysis (glucose, carbohydrates, proteins, fibre)
Biofunctional analysis (phenolic content, antioxidant capacity, melanoidins; and CGA and caffeine by CZE)
Sensorial analysis (20 untrained panellists, hedonic test and acceptance level test)

Study of bioactivity *in vivo*

Effect on body fat accumulation (*C. elegans,* n=120)
Treatment with CSE-beverages, CGA, caffeine and decaffeinated green coffee supplement
Fluorescence (480 nm excitation, 571 nm emission)

NOVEL BAKERY PRODUCTS

CS, CSE, Solid residue

Evaluation of food quality

10 biscuits (with sucrose or maltitol or stevia)
Physical parameters (moisture, thickness, texture and colour)
Food safety (neo-contaminants -acrylamide and HMF- by HPLC)

Study of bioaccesibility *in vitro*

***In vitro* digestion:** Novel biscuit (CSE + solid residue + stevia) and control biscuit (stevia)
Chemical and nutritional composition of digests

SCG

Patent WO2014128320

Validation as food ingredient

Physicochemical characterization
Safety (microbiological quality –aerobic, endospores, moulds and yeasts-, and neo-contaminants - acrylamide and HMF)
Evaluation as dietary fibre (thermal and digestive resistance)

Evaluation of food quality

Biscuits (with stevia or maltitol, SCG and FOS)
Nutritional analysis (proteins and amino acids)
Food safety (microbiological and neo-contaminants– acrylamide, HMF, fructosamine, AGEs)
Sensorial analysis (26 untrained panellists, hedonic test and acceptance test)

Study of bioaccesibility *in vitro*

***In vitro* digestion:** Novel biscuit (SCG + stevia + FOS) control biscuit (sucrose) and food ingredients (SCG, stevia and FOS)
Chemical and nutritional composition of digests

Study of bioactivity *in vitro*

Alpha-glucosidase inhibition
Release of satiety hormones (serotonin and GLP-1 in Caco-2 and HuTu-cells, respectively, by ELISA)

IMPACT OF DIGESTIVE PROCESS ON HEALTH-IMPACT COMPOUNDS

***In vitro* digestion:** Average meal (BSA, digestible starch and oil) and high-sugar meals (BSA, glucose or fructose or HFS)
Intestinal incubation: Simple amino acid systems (lysine or arginine and fructose or glucose)
Determination of MRPs: Amino acid content by amino acid analyzer, fructosamine by NBT assay, CML and MGO-derivative AGEs by ELISA, and fluorescent adducts by fluorescence

Figure 1. Scheme of the work plan performed at the present investigation [ACSE, Arabica coffee silverskin extract; AGEs, advanced glycation end products; BSA, bovine serum albumin; CGA, chlorogenic acid; CML; carboxymethyl lysine; CS, coffee silverskin; CSE, coffee silverskin extract; CZE, capillary zone electrophoresis; ELISA, Enzyme-Linked ImmunoSorbent Assay; FOS, fructooligosaccharides; GLP-1, glucagon-like peptide-1; HMF, hydroxymethyl furfural; HPLC, high-performance liquid chromatography; MGO, methylglyoxal; MRPs, Maillard reaction products; NBT, nitroblue tetrazolium; RCSE, Robusta coffee silverskin extract; SCG, spent coffee grounds]

Main Contributions

MAIN CONTRIBUTIONS

This PhD thesis contributes to provide new knowledge on the validation of CS and SCG as food ingredients in the development of novel beverages and foods. Biological effects of these coffee wastes as food ingredients for the management of obesity and T2D have been examined *in vitro*, *ex vivo* and *in vivo*. The effect of the digestion process on the release of compounds with health impact has been evaluated. Therefore, this section has been divided in 3 chapters as follows: 1) Beverages based on CS; 2) Bakery foods based on CS and SCG; and 3) Impact of digestive process on the formation of potential harmful compounds.

CHAPTER 1

Beverages based on coffee silverskin

This chapter summarises *in vitro* and *in vivo* assays performed to gain insight into the use of CS as a novel beverage and its effects on the obesity disease. *C. elegans* was employed as the animal model to evaluate the reduction of fat deposits.

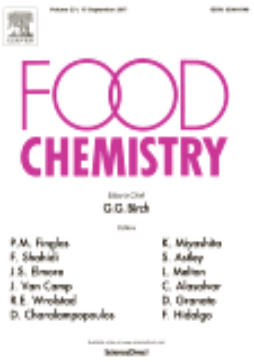
- **Study 1** published as: Martinez-Saez, N.; Ullate, M.; Martin-Cabrejas, M. a.; Martorell, P.; Genovés, S.; Ramon, D.; Del Castillo, M. D. **A novel antioxidant beverage for body weight control based on coffee silverskin**. Food Chem. 2014, 150, 227–234. DOI: 10.1016/j.foodchem.2013.10.100

Study 1: A novel antioxidant beverage for body weight control based on coffee silverskin

Nuria Martinez-Saez[a], Mónica Ullate[a], María A. Martin-Cabrejas[a], Patricia Martorell[b], Salvador Genovés[b], Daniel Ramon[b], María Dolores del Castillo[a]

[a] Institute of Food Science Research (UAM–CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain
[b] Biopolis S.L., Catedrático Agustín Escardino Benlloch 9 Edif 2, 46980 Paterna, Valencia, Spain

Food Chemistry (2014), 150: 227–234
DOI 10.1016/j.foodchem.2016.07.173



Food Chemistry 150 (2014) 227–234

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

A novel antioxidant beverage for body weight control based on coffee silverskin

Nuria Martinez-Saez [a], Mónica Ullate [a], María A. Martin-Cabrejas [a], Patricia Martorell [b], Salvador Genovés [b], Daniel Ramon [b], María Dolores del Castillo [a,*]

[a] Institute of Food Science Research (UAM–CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain
[b] Biopolis S.L., Catedrático Agustín Escardino Benlloch 9 Edif 2, 46980 Paterna, Valencia, Spain

ARTICLE INFO

Article history:
Received 7 March 2013
Received in revised form 24 September 2013
Accepted 23 October 2013
Available online 31 October 2013

Keywords:
Antioxidant
Body weight control
Coffee silverskin
Innovative revalorisation of coffee by-products
Novel beverage

ABSTRACT

The present research aimed to add value to coffee silverskin by looking for new innovative applications. Formulation of novel beverages based on coffee silverskin for body fat reduction and body weight control was proposed. Conditions for beverage preparation were optimised. Data on chemical composition and sensorial quality of the new drink were acquired. Health benefits were evaluated *in vitro* and *in vivo* employing as animal model *Caenorhabditis elegans*. An antioxidant beverage containing physiological active concentrations of caffeine and chlorogenic acid for prevention body fat accumulation and possessing acceptable sensorial properties was obtained. Our findings support that the use of coffee silverskin for obtaining bioactive extract is an innovative way for revalorisation of coffee by-product.

1. Introduction

There has been is a rapid and greatly increased demand for the revalorization of coffee by-products such as coffee silverskin (bean testa, epidermis). Because of its chemical composition, it may be an ideal natural source of a range of compounds with putative health benefits (Borrelli, Esposito, Napolitano, Ritieni, & Fogliano, 2004; del Castillo et al., 2013; Murthy & Naidu, 2012; Murthy, Naidu, & Srinivas, 2009; Napolitano, Fogliano, Tafuri, & Ritieni, 2007). It has been proposed as source of prebiotics (Borrelli et al., 2004; Jaquet, Rochat, Moulin, Cavin, & Bibiloni, 2009), dietary fibre and antioxidants (Borrelli et al., 2004; del Castillo et al., 2013; Napolitano et al., 2007; Narita & Inouye, 2012) as well as inhibitor of hyaluronidase enzyme (Furosawa, Narita, Iwai, Fukunaga, & Nakagiri, 2011) and has already been used as ingredient for formulation of low calorie and high dietary fibre bread (Pourfarzad, Mahdavian-Mehr, & Sedaghat, 2013).

Obesity is increasing worldwide, and among the pathologies associated with this 21th century epidemic, cardiovascular diseases, cancers, metabolic syndrome and diabetes stand out. Consequently, a search for novel foods, drugs or supplements to prevent and treat obesity is a priority worldwide. Several epidemiological investigations associate coffee consumption with weight control. The anti-obesity effect of coffee may be attributed to caffeine (Martin, Clark, Laboy & Davidson, 2010) and chlorogenic acids (CGAs) (Cho et al., 2010; Murase et al., 2011) present in coffee silverskin.

The aim of the present study was to add value to coffee silverskin following an innovative strategy. Its potential as an ingredient in a novel drink for reducing body fat accumulation was evaluated.

2. Materials and methods

2.1. Reagents

Bradford reagent was provided by Bio-Rad Laboratories S.A; glucose kit was from Spinreact (Gerona, Spain); fibre kit from Megazyme International Ireland Ltd. Sulphuric acid 93–98% and ethanol 96% from Panreac S.A. (Spain). Bovine serum albumin (BSA), glucose, caffeine, CGA (3-CGA Reference C-3878), phenol 5% (p/v), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS)), potassium persulphate, Folin–Ciocalteu reagent and Nile Red (9-diethylamino-5H-benzo[α]phenoxazin-5-one) were from Sigma–Aldrich (St. Louis, MO, USA). Water was purified using Milli-Q system. All other chemicals and reagents were of analytical grade.

* Corresponding author. Tel.: +34 91 0017900x953; fax: +34 91 0017905.
E-mail addresses: nur.martinez@titulado.uam.es (N.Martinez-Saez), mullateartiz@ifi.csic.es (M. Ullate), maria.martin@uam.es (M.A. Martin-Cabrejas), patricia.martorell@biopolis.es (P. Martorell), salvador.genoves@biopolis.es (S. Genovés), daniel.ramon@biopolis.es (D. Ramon), mdolores.delcastillo@csic.es (M.D. del Castillo).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.10.100

2.2. Apparatus

A microplate spectrophotometer BioTek powerWaveTM XS (BioTek Instruments, United States), capillary electrophoresis apparatus Agilent G16000A (Agilent, Madrid, Spain) and FP-6200 system (JASCO Analytical Instruments, Easton, MD, USA) were used for analysis.

2.3. Samples

Raw material: Coffee silverskin from Arabic (*Coffea Arabica*) and Robusta (*Coffea canephora*) were provided by Fortaleza S.A. (Spain). Powdered coffee silverskin extracts, Arabic and Robusta, were produced as described in the patent WO 2013/004873. Tablets of a commercial supplement based on decaffeinated green coffee extract and containing CGA (5-caffeoyl quinic acid) as the main bioactive compound were used for comparative analysis.

Beverages: Bags containing samples were prepared using 10–20 μm filter paper and infused in boiling water for different times. Brewing conditions were optimised by studying influence of: (i) raw material (raw coffee silverskin and coffee silverskin extract; (ii) extraction time (5 and 10 min); (iii) concentration of raw material (2.5, 5 y 10 mg/ml) and (iv) coffee variety (Arabic and Robusta). The colour due to melanoidins from the Arabica and Robusta varieties was determined following the procedure described in Section 2.4. Further characterisation of the chemical and nutritional composition of Arabic and Robusta brews was established by employing those methods described in Sections 2.5–2.11. *In vivo* assays and sensory analysis were carried out as described in Sections 2.12 and 2.13 respectively.

2.4. Melanoidins

The content of melanoidins in the brews was estimated by measuring the absorbance at 420 nm. The analysis was performed according to Adams, Borrelli, Fogliano, & De Kimpe, 2005. All measurements were performed in triplicate.

2.5. Caffeine and CGA

The procedure was performed according to del Castillo, Ames, and Gordon (2002). The separation was carried out in a capillary electrophoresis system with an ultraviolet–visible detection. The capillaries used were made with silica and had an internal diameter of 50 μm and a length of 48.5 cm. Separation was performed at 25 °C, a constant voltage of 20 kV, and injection of 50 mbar for 5 s. Detection was performed at wavelengths of 200 nm, 280 nm and 420 nm, and the absorption spectrum recorded in the range 190 and 600 nm. Calibration curves of caffeine (0.25–5.15 mmol/L) and CGA (0.15–2.5 mmol/L) were constructed. The analysis was performed in triplicate. Results were expressed as μg caffeine or CGA/ml.

2.6. Free glucose

Glucose content was determined by using an enzymatic kit as per the manufacturer's instructions (Glucose-TR *in vitro* diagnostic, SPINREACT, S.A.). All measurements were performed in triplicate and the results were expressed as μg glucose/ml.

2.7. Total carbohydrates

Total carbohydrates were determined using the phenol–sulphuric method as described by Masuko et al. (2005). Samples (100 μl) were mixed with 300 μl concentrated sulphuric acid (93–98%) and 90 μl 5% phenol (w/v) in a glass flask. The mixtures were incubated at 90 °C for 5 min. Samples were cooled to room temperature and absorbance was measured at 490 nm. A calibration curve was constructed using glucose (0.1–0.4 mg/ml). Reagent blank and sample blank were also prepared and analysed in each set of samples. All measurements were performed in triplicate and the results were expressed as μg glucose/ml.

2.8. Water soluble proteins

Bio-Rad Protein Assay, catalogue number 500-006, based on the method of Bradford in micro-method format was used to determine protein concentration. Reagents were preparing according to manufacturer's instructions (Bio-Rad Laboratories, SIG 093094). Briefly, a solution of Bradford reagent (1:4 reagent:milli-Q water) was prepared and filtered using Whatman 4 filter papers. 10 μl of the sample and 200 μl of the Bradford solution were placed in a multi-well microplate. The samples were incubated for 5 min at room temperature, and the absorbance was measured at 595 nm. Sample blank and reagent blank were also analysed. A calibration curve was constructed using BSA (0.05–0.5 mg/ml). All measurements were performed in triplicate. Results were expressed as μg BSA/ml.

2.9. Total phenolic compounds

Folin–Ciocalteu adapted to a micromethod format was the test selected for analysis of total phenolic compounds in the samples (Contini, Baccelloni, Massantini, & Anelli, 2008). The reaction was initiated by mixing 10 μl of sample with 150 μl of Folin–Ciocalteu solution. After incubation at room temperature for 3 min, 50 μl of sodium bicarbonate solution were added. The kinetics of the reaction at 37 °C was followed for 120 min by measuring the absorbance at 735 nm once every minute. Sample blank and reagent blank were also analysed in each set of samples. The CGA calibration curve was used for quantification (0.1–0.8 mg/ml). Results were expressed as μmol CGA eq./ml. All measurements were performed in triplicate.

2.10. Total antioxidant capacity

ABTS decolourisation assay was performed according to Oki, Nagai, Yoshinaga, and Nishiba (2006). An $ABTS^{\cdot+}$ stock solution was prepared by adding 140 mmol/L potassium persulfate (44 μl) to a 7 mmol/L $ABTS^{\cdot+}$ aqueous solution (2.5 ml), and the mixture was then allowed to stand for 16 h at room temperature. The working solution of the radical $ABTS^{\cdot+}$ was prepared by diluting the stock solution 1:75 (v/v) in 5 mmol/L sodium phosphate buffer pH 7.4 to obtain an absorbance value of 0.7 ± 0.02 at 734 nm. Samples (30 μl) were added to 270 μl working solution $ABTS^{\cdot+}$ in a microplate. The absorbance was measured at 734 nm for 10 min at 30 °C with measurements every 2 min. After 5 min, the reaction was complete. CGA calibration (0.15–2 mmol/L) was used to calculate overall antioxidant capacity. Results were expressed as μmol CGA eq./ml. All measurements were performed in triplicate.

2.11. Dietary fibre

Insoluble (IDF), soluble (SDF) and total (TDF) dietary fibre content was determined using the Total Dietary Fibre Assay Kit (Megazyme International Ireland, Ireland) as per the manufacturer's instructions, and based on the enzymatic–gravimetric method. Results are expressed as percent (%).

2.12. Body fat reduction in Caenorhabditis elegans: In vivo assays

C. elegans wild type strain N_2 (Bristol), was obtained from the *Caenorhabditis* Genetics Centre (CGC) at the University of Minnesota (USA) and maintained at 20 °C on nematode growth medium. *Escherichia coli* OP50 strain was used as the standard nematode diet and was also provided by the CGC. Compounds were added on the agar surface: CGA (0.01, 0.1, 1 and 10 μmol/L); caffeine (0.025, 0.25, 2.5 and 25 μmol/L). Arabic and Robusta coffee silverskin extracts (25, 50 and 100 μg/ml) were used for a comparative analysis and, in parallel, another comparative study was performed with a commercial supplement and Robusta coffee silverskin extract (65, 130 and 260 μg/ml). Coffee silverskin extract from Robusta variety was selected for comparison with the commercial supplement because of the levels of chlorogenic acids in the samples. Nematodes were fed under different conditions relatives to type and concentration of the samples until the young adult stage. Total lipid content was determined following Martorell et al. (2012) by measuring the fluorescence at 480 nm λ excitation and 571 nm λ emission. Red Nile was used as a dye to monitor lipid storage in live worms. A total of 120 worms for each test were analysed. Experiments were carried out in duplicate.

2.13. Sensory analysis

Sensory analysis was performed using two different tests; a 9-verbal scale test where hedonic attributes were evaluated and an Acceptance Level Test. Sensory evaluation was carried out in one session involving in total 20 untrained panellists allocated to one of two groups. Colour, odour, taste and general acceptance of the selected beverage, A (Arabic coffee silverskin extract 10 mg/ml), B (Robusta coffee silverskin extract 10 mg/ml), C (Arabic coffee silverskin extract 2.5 mg/ml) and D (Robusta coffee silverskin extract 2.5 mg/ml) were tested. Results of the verbal scale test were converted into a 9-point scale scoring 1 (lowest)-9 (highest). For each attribute, the average of the panellists' scores was calculated. Beverages were considered acceptable if their mean value for overall quality scores was equal or above 5 (neither like nor dislike).

2.14. Statistical analysis

Data were expressed as mean ± standard deviation (SD). *T*-test (independent samples, 2 groups) and Analysis of Variance (more than 2 groups), one-way and two-way ANOVA, were applied to determine differences between means. Differences were considered to be significant at $p < 0.05$, highly significant $p < 0.01$ and very highly significant $p < 0.001$. Relationships between the different parameters analysed were evaluated by computing Pearson linear correlation coefficients at the $p < 0.05$ confidence level.

3. Results and discussion

3.1. Selection of raw material for brewing

3.1.1. Melanoidins

Colour due to coffee melanoidins was estimated by measuring the absorbance values at 420 nm. Values corresponding to silverskin extracts (Fig. 1) were significantly higher ($p < 0.05$) than those corresponding to raw silverskin. Melanoidins are the final product of the Maillard reaction formed during the coffee bean roasting process. The complex and variable structure of coffee melanoidins is basically formed by polysaccharides, proteins and chlorogenic acids. Coffee melanoidin fractions are diverse and they possess different physicochemical properties. It is known that a fraction of coffee melanoidins present anionic character whereas other

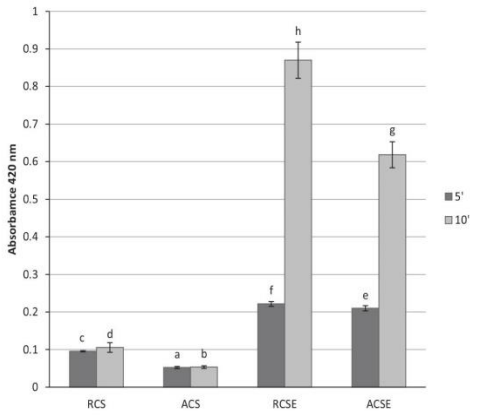


Fig. 1. Release of melanoidins in beverages elaborated with Robusta coffee silverskin (RCS), Arabic coffee silverskin (ACS), Robusta coffee silverskin extract (RCSE) and Arabic coffee silverskin extract (ACSE) (2.5 mg/ml) using 5 and 10 min of extraction. Bars represent the mean values ($n = 3$), and the error bars the standard deviation. Different letters indicate significant differences between the means of the samples ($p < 0.05$).

fractions do not present it or present it to a very small extent. In addition, there are coffee melanoidin fractions presenting hydrophobic character (Moreira, Nunes, Domingues, & Coimbra, 2012). Results shown in Fig. 1 were as expected. Silverskin extract is enriched in coffee melanoidins soluble in hot water. In agreement, higher concentrations of melanoidins should be present in samples prepared with extracts compared to those based on raw silverskin. A significant association was observed between the concentration of melanoidins and extraction time ($p < 0.05$). Results indicate that treatment in boiling water for 10 min was adequate to achieve high solubility of most of coffee melanoidins present either in extracts and raw silverskin.

Silverskin melanoidins may provide taste, aroma and colour to the beverage. From the bioactivity point of view, melanoidins in coffee have many beneficial properties to offer such as antioxidant, antimicrobial, anticariogenic, anti-inflammatory, antihypertensive and antiglycative (Moreira et al., 2012). Other studies have shown that weight gain in rats fed on a diet rich in Maillard reaction products is lower than a matched control group (Šebeková et al., 2012). Therefore, the presence of these compounds in the new beverage may be assessed positively, as they can contribute to the product's sensorial acceptability and its biofunctional properties. Silverskin extracts may give rise to a product with more intense sensorial effects and greater biological activity than those made from silverskin because they contain higher melanoidins concentrations than those with only raw silverskin.

3.1.2. CGA and caffeine content

As expected, the amount of silverskin extract had a significant effect ($p < 0.05$) on caffeine and CGA content of brews (Fig. 2A and B). The lowest concentrations of bioactive compounds were detected in the Arabic coffee silverskin extract samples, with a concentration of 2.5 mg/ml. In comparison, the highest values were found in brews prepared with Robusta coffee silverskin extract, with 10 mg/ml. These results suggest that Robusta coffee silverskin extract is a better source of CGA and caffeine than Arabic coffee silverskin extract.

Currently available data on the chemical composition of coffee silverskin is scarce. Caffeine values between 0.83 and 1.37 g/100 g of silverskin (Napolitano et al., 2007) and CGA in the

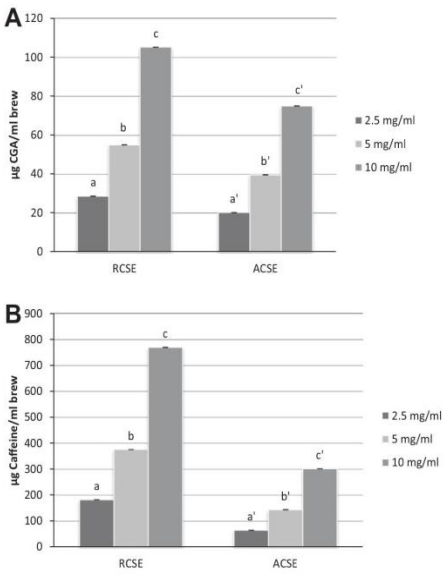


Fig. 2. CGA (A) and caffeine (B) concentrations, expressed as mg CGA/ml brew and µg caffeine/ml brew, in beverages prepared with Robusta coffee silverskin extract (RCSE) and Arabic coffee silverskin extract (ACSE) at three different concentrations, 2.5, 5 and 10 mg/ml, using 10 min of extraction. Bars represent the mean values ($n = 3$), and the error bars the standard deviation. Different letters denote significant differences between the means ($p < 0.05$) within a set of samples.

region of 1.10 mg/100 g (Borrelli et al., 2004) to 3 g/100 g (Murthy & Naidu, 2012) have been reported. The values found in the samples analysed in this study show that the extraction protocol patented by our research group has allowed us to obtain extracts enriched in bioactive compounds, which may allow us to obtain beverages with physiologically active concentrations of those compounds (WO 2013/004873). Extracts were obtained containing 751.20 mg/100 g and 3.02 g/100 g of CGA and caffeine respectively in Arabic coffee silverskin extract and 1053.80 mg CGA/100 g and 7.70 g caffeine/100 g in Robusta coffee silverskin extract. The effect of caffeine and CGA on the metabolism have previously been described for both compounds independently and in combination with other compounds as well as for coffee alone (Glade, 2010; López-García et al., 2006; Murase et al., 2011; Mure et al., 2013).

3.2. Nutritional characterisation of the new beverage

3.2.1. Nutrients

Table 1 shows data on nutrients for brews prepared from Robusta and Arabic coffee silverskin extracts at 2.5 mg/ml and 10 min extraction. No glucose was detected in these beverages. Conversely, in beverages made from Arabic coffee silverskin extract 10 mg/ml, free glucose values were 12.83 ± 6.01 mg/ml and this compound was not detected in the beverage made with Robusta coffee silverskin extract (data not shown). Total carbohydrate levels in the samples differed significantly ($p < 0.05$) with the highest levels of these compounds found in the beverages made from Arabic coffee silverskin extract. Additionally, statistically different results were collected ($p < 0.05$) for extractable protein content, with the highest concentration of this nutrient found in the Robusta sample.

The results for reducing sugars, which are shown in Table 1, correspond to those described by Borrelli et al. (2004) and Napolitano et al. (2007). These authors described null or low concentrations of reducing sugars in coffee silverskin. However, the total carbohydrate and protein levels found in their research were higher than those we detected. Earlier studies suggest that coffee silverskin is a product rich in polysaccharides, mainly arabinogalactans and galactomannans (Borrelli et al., 2004; Napolitano et al., 2007). The highest values encountered for total carbohydrates and proteins in our brews were 13.42 and 5.37 g/100 g, respectively. The differences may be due to the extraction process used. Complex water-insoluble proteins and carbohydrates may have remained integrated in the vegetable matrix that is insoluble in the extraction treatment considered and, thus, disposed of as part of the solid waste resulting from the extraction process.

Table 1
Values of total carbohydrates, glucose and proteins, in Robusta coffee silverskin extract (RCSE) and Arabic coffee silverskin extract (ACSE) brews, 2.5 mg/ml and 10 min of extraction.

Measurement	Variety	
	RCSE	ACSE
Glucose (µg glucose/ml)	n.d.[A]	n.d.[A]
Total carbohydrates (µg glucose/ml)	136.88 ± 6.28[a]	335.73 ± 33.37[b]
Water soluble protein (µg BSA/ml)	134.29 ± 3.95[b]	24.87 ± 1.22[a]

Each value represents the mean ($n = 3$) ± standard deviation. Different letters denote significant differences ($p < 0.05$) between samples of the same row.
[A] Non-detected.

According to the results shown in Table 1, the beverages obtained may be included in the diet of patients with carbohydrate and energy metabolism problems, as in the case of patients with obesity and diabetes because its free glucose content. The new beverage may be expected to present low glycaemic levels as in the case of the beverage coffee, which is considered to have a low or null glycaemic index (http://www.montignac.com).

3.2.2. Dietary fibre

Coffee silverskin extract as raw material for preparation of the new beverage showed quantities of TDF ranging from 23% to 19% (w/w) for Arabic and Robusta coffee silverskin extract, respectively (data not shown). In both cases, soluble fibre content was greater than insoluble fibre content, with a SDF/IDF ratio of 2.3 for Arabic coffee silverskin extract and 1.8 for Robusta coffee silverskin extract, respectively.

According to the literature, SDF content (~9%) is comparable to that of oat bran, which is considered as a rich source of this dietary compound (Borrelli et al., 2004). Studies on dietary fibre composition of coffee by-products by Murthy and Naidu (2012) suggest that coffee silverskin is the coffee by-product containing the highest quantities of TDF (80%).

Data on TDF in coffee by-products are similar to those described for cereals and vegetables (Figuerola, Hurtado, Estévez, Chiffelle, & Asenjo, 2005). The values found by our research group are lower than those found by other authors employing direct analysis of coffee silverskin. The results indicate that despite such differences, which may be accountable to the extraction process, among other factors, the extract obtained and employed as a raw material for manufacturing the new beverage is a source rich in dietary fibre and especially SDF. These results strengthen the hypotheses that the new beverage could feature a low glycaemic index and be appropriate for the prevention and/or treatment of conditions related to energy and/or carbohydrates metabolism and effective body weight control.

Dietary fibre from coffee is thought to be composed chiefly by cellulose, hemicellulose, pectic substances and lignin (Borrelli et al., 2004), comprising the monosaccharides glucose (cellulose) and xylose, galactose, mannose, arabinose (hemicellulose) (Mussatto,

Machado, Martins, & Teixeira, 2011). Antioxidant properties are associated to coffee silverskin fibre, attributed to the presence of CGA adhering to the dietary fibre matrix formed by a complex carbohydrate structure. Consequently, it has been classified as antioxidant dietary fibre (Murthy & Naidu, 2012; Napolitano et al., 2007).

SDF and IDF present different physiological functions. Studies have shown that SDF is capable of reducing the absorption of macronutrients and slows down gastric emptying, which reduces the postprandial glucose response, total cholesterol levels and low-density lipoproteins (LDL) (King et al., 2007). Moreover, increasing soluble and insoluble fibre intake significantly reduces the risk of gaining weight and fat in women, independent of several potential confounders, including physical activity, dietary fat intake, and others. The influence of fibre seems to occur primarily through reducing energy intake over time (Tucker & Thomas, 2009).

On the basis of the results of our research and those described by other authors it can be considered that the beverages made from Robusta and Arabic coffee silverskin extracts are both potentially of interest in body weight control and prevention of obesity and diabetes. Recommendations with regard to dietary fibre consumption vary depending on the regulatory body issuing them. Thus, for example, the World Health Organization (WHO) recommends a daily intake of 27–40 g of TDF; the U.S. Food and Drug Administration (FDA) proposes 25 g of fibre per day for each 2000 kcal/day in the case of adults, while the American Dietetic Association (ADA) recommends between 20 and 30 g/day of fibre, of which 3–10 g should be SDF (15–30% soluble fibre). The consumption of one cup (200 ml) of coffee silverskin extract beverage with a concentration of 10 mg/ml can provide approximately 0.40 g of TDF and 0.25 g of SDF. Moderate consumption of this beverage, consisting of 3 cups a day, which is comparable to the recommendation for coffee, can provide 10% of the daily average amount of SDF recommended by the ADA. It is important to underscore that not all foods are rich in this type of fibre, which has special physiological properties that may be beneficial in controlling body weight. This rate of consumption would be equivalent to drinking approximately 300 mg of caffeine and 55 mg of CGA a day. The caffeine intake from drinking the beverage prepared as described would be equivalent to the amount present in instant coffee. There are not safety limits established for exposure to caffeine. However, earlier studies suggest that consuming 400 mg/day does not cause adverse effects on adults, and the accepted recommended maximum for caffeine consumption in adults is about 3 mg/kg of body weight (http://www.happily-healthy.com.au/caffeine-knowing-the-facts-2). As a consequence, the beverage prepared as described could have positive physiological effects with potentially no adverse effects on consumers' health. More research is needed to test this hypothesis.

3.3. Biofunctional characterisation of the new beverage

3.3.1. Total antioxidant capacity in vitro

As shown in Fig. 3, while all the beverages had antioxidant capacity, levels were significantly greater ($p < 0.05$) in beverages made with Robusta coffee silverskin extract than Arabic. The same trend was observed applying both of the methods. The correlation test on the data obtained using both methods showed a correlation coefficient close to 1 ($p < 0.05$). The results agree with those described by Napolitano et al. (2007) and del Castillo et al. (2013) who found, in general, a higher proportion of extractable antioxidants in aqueous solution for the samples of Robusta silverskin, in comparison with Arabica samples. Moreover, the beverages made from Robusta and Arabic coffee silverskin extracts, both

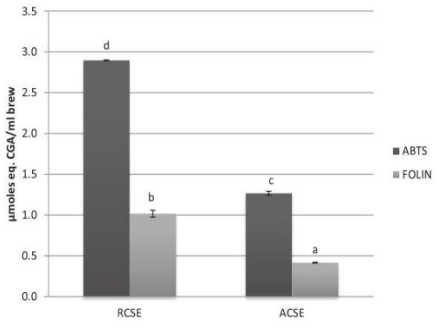


Fig. 3. Data obtained by $ABTS^{\cdot+}$ and Folin–Ciocalteu methods. The beverages made with Robusta coffee silverskin extract (RCSE) and Arabic coffee silverskin extract (ACSE) were prepared at a concentration of 2.5 mg/ml, and 10 min of extraction. Results are expressed as µmoles eq. CGA/ml brew. Bars represent the mean values ($n = 3$) whereas the error bars show the standard deviation of the mean of measurements in triplicate. Different letters denote significant differences between the means ($p < 0.05$).

presented total antioxidant capacities similar to those described for coffee beverage and coffee silverskin (Borrelli et al., 2004).

The differences found in the total antioxidant capacity values in the Arabica and Robusta beverages (Fig. 3) correspond with those detected by analysing their components, melanoidins and CGA (Figs. 1 and 2A). Earlier studies suggest that these compounds contribute to the antioxidant properties of coffee silverskin (del Castillo et al., 2013; Murthy & Naidu, 2012; Napolitano et al., 2007). The beverages made from these extracts contained CGA at 0.09 and 0.06 µmol/ml at a concentration of 2.5 mg/ml Robusta and Arabica, respectively (Fig. 2A). These values are lower than those found by the Folin and ABTS decolourisation methods (Fig. 3). We conclude that although the Folin method was used to determine free phenolic compounds, other components in the beverage may have reacted with the reagent interfering in the final measurement. The preparation of samples for this assay did not involve any prior extraction of phenolic compounds. Moreover, previous studies indicate a lack of selectivity in the method and suggest compounds where CGA may be present, such as melanoidins, may also be determined using this method (del Castillo et al., 2013; Murthy & Naidu, 2012; Napolitano et al., 2007). Therefore, the Folin reaction seems to be providing information on the concentration of CGA, free and linked to complex structures (melanoidins and antioxidant fibre) present in the beverages. These data are supported by those obtained by the ABTS radical cation decolourisation method. In view of the results available, it can be stated that about 3–4% of the total antioxidant capacity of the beverages may be due to free CGA. The low concentrations of free CGA in the sample are due to the fact that the extract is made from a by-product of coffee bean roasting. This procedure has a dramatic effect on the content of those compounds naturally present in the beans, such as CGAs, and gives rise to the formation of neoantioxidants deriving from the Maillard reaction, principally melanoidins (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002; Vignoli, Bassoli, & Benassi, 2011). There is proof, however, of the incorporation of CGA structures, caffeic and quinic acid in the dietary fibre and melanoidins (Moreira et al., 2012; Silvan, Morales, & Saura-Calixto, 2010).

In summary, the results indicate that total antioxidant capacity of the beverage is an indirect indicator of the concentration of compounds with different bioactivity such as CGA, melanoidins and dietary fibre. A number of studies associate CGA and coffee melanoidins with reduction of body weight, indispensable in the prevention and treatment of obesity (Cho et al., 2010; Murase et al., 2011; Šebeková et al., 2012). Therefore, measuring this property

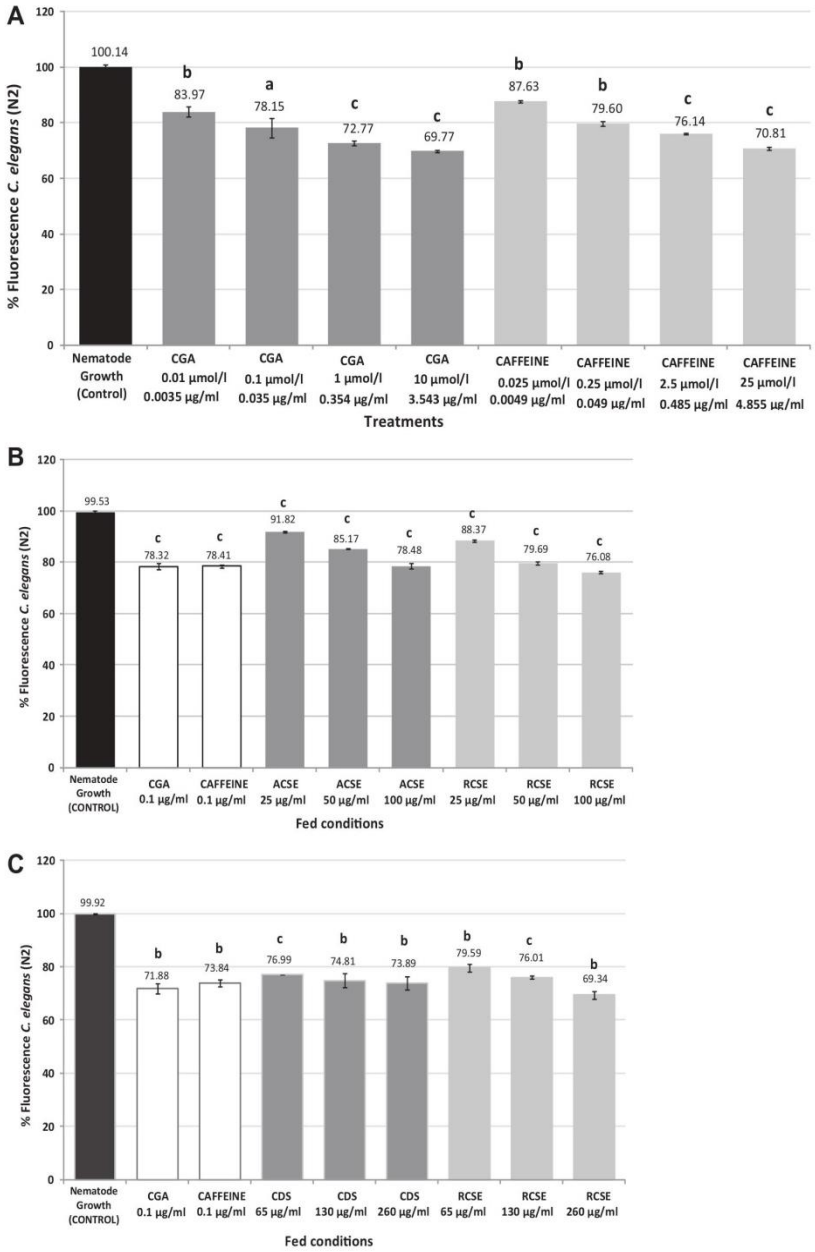


Fig. 4. Percentages of fluorescence obtained in *C. elegans* wild type strain (N2) at young adult stage. (A) Dose–response assay with CGA (0.01–10 µmol/L) and caffeine (0.025–25 µmol/L). (B) Comparison between Arabic (ACSE) and Robusta coffee silverskin extract (RCSE) brews at 25, 50 and 100 µg/ml respectively. (C) Nematodes were fed with a commercial dietetic supplement (CDS) or Robusta coffee silverskin extract (RCSE) brews at three different concentrations (65, 130 and 260 µg/ml). Pure compounds, CGA (0.1 µg/ml) and caffeine (0.1 µg/ml) were also included as positive controls in the experiments. (a) Significant p-value $\leqslant 0.05$; (b) significant p-value $\leqslant 0.01$; (c) significant p-value $\leqslant 0.001$.

may be an appropriate index for tracing the stability of foods, their composition, and forecasting their potential effect on body weight control.

3.3.2. Effect of the new beverage on body fat content in vivo

The results obtained by quantification of fluorescence in each population of nematodes (wild type N2 *C. elegans*) for pure CGA

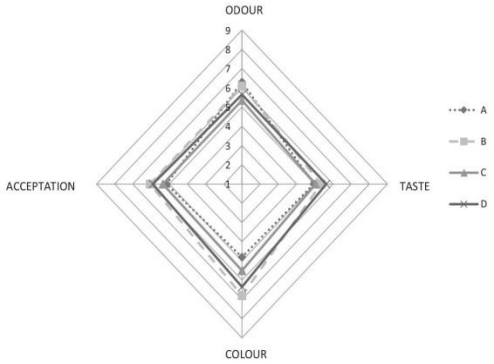


Fig. 5. Spider-web diagram which shows the mean scores ($n = 20$) of 1–9 scale, for each of the attributes and beverages.

compounds (0.01–10 μmol/L) and caffeine (0.025–25 μmol/L) showed a very clear significant dose–response effect on reducing accumulation of body fat, with the reduction increasing as we increased the concentration of the compound (Fig. 4A). The highest values for reducing the percentage of body fat obtained were 30% and 29%, for CGA (10 μmol/L) and caffeine (25 μmol/L) respectively. The results also suggest that CGA has a greater fat reducing effect than caffeine in the animal model studied.

A highly significant decrease in fluorescence was observed with the brews containing Arabica and Robusta silverskin extracts ($p \leqslant 0.001$) (Fig. 4B). All three concentration levels analysed (25, 50 and 100 μg/ml) gave rise to body fat reducing phenotypes. This may highlight the dose–response effect of both extracts, and lower levels of fluorescence, an indicator of fat accumulation, were observed as the extract dose increased in the cultivar medium. The most effective dose was found to be 100 μg/ml, corresponding to the beverage of 10 mg/ml (body fat reduction of 21% and 24% for Arabic and Robusta coffee silverskin extract, respectively). The beverages made from Arabic and Robusta coffee silverskin extracts in this study are equivalent to concentrations in *C. elegans* growth medium of 0.81–3.22 μmol/L and 1.63–6.5 μmol/L of CGA and 3.35–15.6 μmol/L and 9.42–39.7 μmol/L of caffeine, respectively, which are within the range of bioactivity observed for pure compounds (Fig. 4A and B). Therefore, it can be considered that they contain physiologically active doses of these compounds and may have an effect in the prevention of obesity.

The results seem to indicate that the CGA and caffeine compounds simultaneously present in the beverages might exert a synergic and/or additive effect on fat reduction. However, individual pure compounds and those naturally present in the food matrix lead to different fat reduction values. Such differences may be explained by the food matrix effect. Complex molecules such as dietary fibre, proteins and melanoidins in the composition of the beverages could affect the bioavailability of CGA and caffeine, respectively. More research is necessary to confirm this hypothesis. Additionally, a functional effect was observed (Fig. 4B) slightly greater with the beverages made from Robusta coffee silverskin extract than in those made from Arabic. This may be a consequence of the higher concentration levels of CGA and caffeine present in the Robusta coffee silverskin extract (Fig. 2A and B).

The comparison between the commercial supplement made from Robusta decaffeinated green coffee extract and the beverage made from Robusta coffee silverskin extract is shown in Fig. 4C. It was observed that body fat reduction increased as we raised the concentration of the product. Furthermore, similar results were observed for body fat reduction from both Robusta coffee silverskin extract beverages and the commercial dietary supplement.

Concentration levels of 0.28 μmol/L of pure CGA compound (3-CGA), equivalent to 0.1 μg/ml, have similar effects to those registered for concentration assays on the supplement (Fig. 4C). The results suggest that this commercial product is less effective than the pure compound. The minimum CGA concentration, supplemented in the diet with the addition of commercial green coffee extract, was twice as high as the concentration of pure CGA (3-CGA) required for the same effect. As highlighted previously, caffeine is not present in the supplement and, from the results, it was shown that CGA bioavailability can be affected by other compounds in the extract, and CGA, 3-CGA and 5-CGA isomers may have different degrees of bioactivity. The possibility that other compounds in the supplement may also be interfering with the bioactivity can be ruled out.

Therefore, it can be stated that the new beverage made from roasted coffee silverskin extract, under the assay conditions in this study, showed biofunctional qualities comparable to the commercial dietary supplement in terms of body fat reduction. It is a natural alternative to dietary supplements for the prevention of excess weight and obesity.

Several other research studies have been found that illustrate the usefulness of the *C. elegans* model for gathering pre-clinical data on alimentary products similar to the new beverage proposed herein (Dostal, Roberts, & Christopher, 2010; Surco-Laos et al., 2012).

3.4. Sensorial analysis of the new beverage

As shown in Fig. 5, all assayed attributes were given a grade equal to or higher than five. As regards colour, beverage B (Robusta coffee silverskin extract 10 mg/ml) received the highest points, with an average of 7, followed by beverage D (Robusta coffee silverskin extract 2.5 mg/ml). Beverages B and D were the most palatable in terms of taste with the Robusta beverages scoring more higher. Beverages A (Arabic coffee silverskin extract 10 mg/ml) and B were the most attractive in terms of smell, which seems to be related to beverage concentration. Beverages with a concentration of 10 mg/ml scored the highest. These results matched expectations, since the concentration of aromatic compounds determines the beverage's aroma. As expected beverages B and D, made from Robusta coffee silverskin extract, scored the highest points overall amongst the samples tested with a grade. This does not mean, however, that beverages made from Arabic coffee silverskin extract were found distasteful by the panel.

Moreover, regarding the Acceptance Test, it was found that 10% of the testing panel were prepared to consume the beverages as served, 85% would drink them but add another ingredient (sugar, milk, citrus, ice, etc.) and 5% declared they would not drink them. Therefore, we can conclude the acceptance level was satisfactory as 95% of the panel were favourable towards the beverages before being told about the benefits of consumption. Beverage B, made from Robusta coffee silverskin extract 10 mg/ml, which achieved the highest acceptance score in all parameters measured, also showed the highest levels of bioactivity *in vitro* and *in vivo* (Figs. 4 and 5).

The influence of the variables (i) gender, (ii) habitual consumption of caffeinated beverages, and (iii) presence or absence of other added ingredients (sweetener, lemon or milk) on the product's appeal was negligible ($p > 0.05$).

According to the literature on the subject, Arabic coffee is characterised by better quality beans producing fine, aromatic coffee. Robusta coffee, on the other hand, produces a less aromatic coffee, rougher and stronger in flavour (Capel & Pérez, 2010). Albanese, Di Matteo, Poiana, and Spagnamusso (2009), in a study

on the influence of sensorial attributes in *espresso* coffee, observed that samples containing a high percentage of Robusta coffee in the blend were characterised by acid notes, bitter taste and astringent properties. Despite this, the sensorial analysis carried out in this study indicates a higher degree of acceptance for the beverages made from Robusta rather than from Arabica silverskin extract.

4. Conclusion

An antioxidant beverage with right nutritional composition, sensorial quality and potential for preventing fat accumulation and excess weight may be formulated by employing coffee silverskin extract. The characteristically chemical and nutritional composition of the extract supports its potential for other health promoting applications.

Acknowledgements

This work has been funded by Naturage (AGL2010-17779) and CONSOLIDER Ingenio 2010 (FUN-C-FOOD: CSD 2007-00063) projects. Nuria Martínez-Sáez thanks U.A.M. for her research fellowship. Authors also thank Dr. P. Martin for the statistical analysis of the data and Cafinsa, Fortaleza, Vitoria for supplying the raw material for preparation of bioactive extracts.

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CHAPTER 2

Bakery foods based on coffee silverskin and spent grounds

The present chapter shows findings regarding the validation of CS and SCG as food ingredients of bakery products. Physical, nutritional, sensorial and safety qualities are presented. Evidence on the bioaccesibility *in vitro* of these bakery foods is shown. Effects of the bioaccessible food components released during the *in vitro* simulated human digestion of a novel coffee fibre-containing biscuit (CFB) on α-glucosidase activity and satiety hormones, serotonin and GLP-1, are shown. The satiating effect was evaluated *ex vivo* in gut intestinal cells (Caco-2 and HuTu-80).

- **Study 1** published as: Garcia-Serna, E.; Martinez-Saez, N.; Mesias, M.; Morales, F. J.; del Castillo, M. D. **Use of coffee silverskin and stevia to improve the formulation of biscuits**. Polish J. Food Nutr. Sci. 2014, 64, 243–251. DOI: 10.2478/pjfns-2013-0024.

- **Patent:** del Castillo, M. D.; Martinez-Saez, N.; Ullate, M. **Formulación alimentaria que comprende marros de café y sus aplicaciones**. WO2014128320 A1 2014 (***ANNEX 1***).

- **Study 2** published as: Martinez-Saez, N.; Tamargo, A.; Domínguez, I.; Rebollo-hernanz, M.; Mesías, M.; Morales, F. J.; Martín-Cabrejas, M. A.; del Castillo, M. D. **Use of spent coffee grounds as food ingredient in bakery products**. Food Chem. 2017, 216, 114–122. DOI: 10.1016/j.foodchem.2016.07.173.

- **Study 3** will be published as: **Martinez-Saez, N.;** Hochkogler, C. M.; Somoza, V. & del Castillo, M.D. ***In vitro* assessment of the satiety effect of non-added sugar biscuit containing stevia, coffee fibre and fructooligosaccharides**. This article has been submitted to Nutrients on the 27th of April, 2017.

Study 1: Use of coffee silverskin and stevia to improve the formulation of biscuits

Esther Garcia–Serna[1], Nuria Martinez–Saez[1], Marta Mesias[2], Francisco J. Morales[2], M. Dolores del Castillo[1]

[1] Institute of Food Science Research (CSIC–UAM), C/ Nicolás Cabrera, 9, Campus de Cantoblanco, Universidad Autónoma de Madrid, 28049 Madrid, Spain, www.cial.uam–csic.es
[2] Institute of Food Science and Technology and Nutrition (ICTAN–CSIC), C/ José Antonio Novais, 10, 28040 Madrid, Spain, www.ictan.csic.es

Polish Journal of Food and Nutrition Sciences (2014), 64 (4): 243–251. *DOI: 10.2478/pjfns-2013-002*

Polish Journal of Food and Nutrition Sciences
DE GRUYTER OPEN

Pol. J. Food Nutr. Sci., 2014, Vol. 64, No. 4, pp. 243–251
DOI: 10.2478/pjfns-2013-0024
http://journal.pan.olsztyn.pl

Original article
Section: Food Quality and Functionality

Use of Coffee Silverskin and Stevia to Improve the Formulation of Biscuits

*Esther Garcia–Serna[1], Nuria Martinez–Saez[1], Marta Mesias[2], Francisco J. Morales[2], M. Dolores del Castillo[1]**

[1]Institute of Food Science Research (CSIC–UAM), C/ Nicolás Cabrera, 9, Campus de Cantoblanco, Universidad Autónoma de Madrid, 28049 Madrid, Spain, www.cial.uam–csic.es
[2]Institute of Food Science and Technology and Nutrition (ICTAN–CSIC), C/ José Antonio Novais, 10, 28040 Madrid, Spain, www.ictan.csic.es

Key words: stevia, coffee silverskin, acrylamide, HMF, quality, biscuits, low sugar, maltitol

Decreasing the amount of sugar added to biscuits is a good strategy to obtain a healthy product. However, a reduction in the quantity of sugar may affect its nutritional value and quality. The feasibility of the combined use of stevia and coffee silverskin for achieving healthier, nutritious and good quality biscuits has been investigated. Ten wheat flour biscuit formulations were designed. Sucrose, maltitol and stevia were used as sweeteners and coffee silverskin was used as a natural colouring and as a source of dietary fibre. The quality of the biscuits was evaluated by measuring their moisture, thickness, breaking force and colour. Acrylamide (ACR) and hydroxymethylfurfural (HMF) contents were also determined in the interest of food safety. The quality and safety of the innovative biscuits was obtained by an analysis of the sugars, proteins, free amino acids, chlorogenic acid, overall antioxidant capacity and acrylamide after *in vitro* digestion. Only the stevia biscuits and those added with coffee silverskin extract and the solid residue recovered from the extraction process, were selected for that study. A comparison of the stevia formulated biscuits, with the stevia formula added with silverskin, showed that the added biscuits had a good nutritional quality and improved texture and colour.

INTRODUCTION

Nowadays, food industry is searching for solutions to reduce the levels of certain ingredients in their products, such as salt, fats and sugars, in order to produce healthier foods that meet the consumers' expectations. High sucrose levels are associated with health problems such caries, obesity, type II diabetes, high blood cholesterol and coronary diseases. During the baking process complex, biochemical and physicochemical reactions occur, which can affect the quality and safety of the biscuits. Sucrose is the main sugar used in the biscuit industry, and it plays an important role in the manufacturing process and in the final quality of the product. Sugar determines the gelatinisation of starch, gluten mobility, biscuit spread, crispness and the surface characteristics of baked biscuits. Consequently, sugar affects the flavour, dimensions, colour, hardness, and surface of the final product [Laguna *et al.*, 2013]. On the other hand, contaminants may be formed during food processing, *e.g.* Maillard reaction products such as acrylamide (ACR) and hydroxymethylfurfural (HMF) [Kukurová *et al.*, 2013; Morales, 2009; Pedreschi *et al.*, 2014]. Therefore, decreasing the quantity of sugar added to biscuits might be a good strategy of obtaining a healthy and a low sugar product.

Sugar replacement is a challenge for the food industry because of the many functions it has in biscuit manufacture. Polyols like maltitol are suitable as sugar replacements [Martinez–Cervera *et al.*, 2014] because they do not affect the sensory properties or mitigate the formation of HMF [Courel *et al.*, 2009; Morales, 2009]. However, no data regarding the effect of maltitol on acrylamide formation are available.

Stevia, in particular its steviol glycosides, have beneficial effects on human health such as a low calorific content, antioxidant properties, an anti–diabetic capacity [Sharma *et al.*, 2012] as well as renal protective characteristics [Shivanna *et al.*, 2013]. Steviol glycosides are an authorised food additive (EU Regulation 1131/2011) and their popularity is rapidly increasing. Substituting sucrose with stevia (*Stevia rebaudiana Bertoni*) inhibits acrylamide formation and has an effect on dough rheology and the baking attributes of biscuits [Abdel–Shafi *et al.*, 2011]. Substitution by more than 30% of the sugar with stevia also alters quality attributes such as moisture content, colour development, spread ratio, breaking force, and consequently the sensory acceptance of the biscuits.

Coffee silverskin can be used in the preparation of functional beverages [Martinez–Saez *et al.*, 2014] and bakery products [Pourfarzad *et al.*, 2013] with acceptable sensorial quality. Adding dietary fibre to the biscuit formulation has an impact on its rheological properties, water activity, viscosity, sensorial quality and nutritional properties [Popov–Raljic

* Corresponding Author:
Tel. +3491 0017900 Ext. 953; Fax: +3491 0017905;
E–mail: mdolores.delcastillo@csic.es (Dr. M. Dolores del Castillo)

et al., 2013]. Coffee silverskin, is a by–product of roasting coffee, rich in dietary fibre, which makes it a good candidate for improving the overall quality of stevia biscuits. No studies regarding the impact of coffee silverskin addition on the HMF and ACR content, colour and textural parameters of biscuits are available.

The present research aims to evaluate the usefulness of coffee silverskin and stevia for obtaining healthier, safer, and high quality dietary biscuits. The effect of replacing sucrose with maltitol on the formation of acrylamide has also been studied. The quality properties of the novel food, the maltitol and sucrose biscuits, have been compared. The bio–accessibility of the nutrients, ACR and the functional components has been determined in order to evaluate the balance between the risks and benefits of the new formulation.

MATERIALS AND METHODS

Reagents

Pepsin, α–amylase, pancreatin, bile extract, bovine serum albumin (BSA), starch, potassium persulfate, chlorogenic acid (CGA, Reference C–3878), 6–hydroxy2,5,7,8–tetrametilhylchroman–carboxilicoylic acid (Trolox), and N–α–acetyl––L–lysine, were supplied by Sigma–Aldrich. Ethanol (96% v/v) and formic acid were supplied by Panreac S.A. (Spain). 2,2'–azino–bis (3–ethylbenzothiazoline–6–sulphonic acid (ABTS)), reagent o–phthaldialdehyde (OPA) and sodium dodecyl sulfate (SDS) were supplied by Fluka and β–mercaptoethanol by Merck (Germany). Bio–Rad Laboratories S.A provided Bradford reagent. The ACR marker ($^{13}C_3$ – acrylamide) (isotopic purity 99 %) was provided by Cambridge Isotope Labs (USA). Methanol and acetonitrile (HPLC grade) were provided by Scharlau (Spain). Cartridges for solid phase extraction – Oasis HLB (30 mg, 1 mL) from Waters (USA) and fibre kit from Megazyme International Ireland Ltd. D–glucose, mannose and fructose kit were supplied by Megazyme (Ireland). All other chemicals and reagents were of analytical grade.

Food ingredients

All the basic ingredients were purchased at specialised and certified food markets. Food grade soy lecithin was provided by Manuel Riesgo SA (Spain) and maltitol was supplied by a national food company. The commercial stevia sweetener powder which contained 3% steviol glycosides, was supplied by Gerblé (Spain).

Arabica coffee silverskin was provided by Fortaleza S.A. (Spain). The extract, which contained natural colouring as well as other bioactive compounds, was obtained using the method described by del Castillo *et al.* [2013].

Briefly, 3.3 g of silverskin was treated with 100 mL boiling water for 10 min. The solid residue from the extraction process was recovered by filtration using gravimetric paper filters and dried at room temperature for 24 h. The dried solid was used as natural source of dietary fibre. Addition of raw coffee silverskin was also carried out. The total dietary fibre of coffee silverskin was determined by the enzymatic gravimetric method using the Total Dietary Fibre Assay Kit (Megazyme International Ireland, Ireland).

Preparation of biscuits

A total of ten biscuit formulations were prepared as described in Table 1. The dough was prepared by mixing salt, baking powder and sugar or its substitutes. Mineral water at room temperature was added to the dry mixture and thoroughly blended to obtain a homogenous mixture. In a separate bowl, lecithin and oil were mixed and then added to the mixture. Finally, the flour was added gradually to the mixture and the dough was kneaded to obtain a homogeneous, elastic and slightly sticky dough. The dough was allowed to rest for 30 min, and shaped into discs with a diameter of 6.4 cm and a thickness of 0.8 cm. The surface of the biscuits was punctured several times using a fork to prevent puffing. In those formulations with coffee silverskin or the solid residue from the extraction process, these were combined with the flour and added as described above. Coffee silverskin extract was used as a substitute for water in the biscuit formulation.

The biscuits were baked at 190ºC for 20 min with air recirculation (30% power) in a Memmert GmbH (Schwabach, Germany) UNE–400 model oven. Two sets of 4 biscuits were baked in duplicate (n=8). The biscuits were placed in the centre of the tray forming a square, in order to reduce process variability during baking. The temperature of processing was controlled and monitored by internal sensors (2007 Celsius v8.0, Memmert GmbH) and externally using temperature controller (type K, 0.1 mm, 0.1ºC accuracy), located at tray height. A temperature measurement was recorded every second in a data logger (Delta OHM, Model HD–2178–2, Italy) throughout the process.

Quality attributes

Moisture

Prior to analysis, the capsules were heated at 110ºC for 15 min, cooled in a desiccator at room temperature. The moisture content was determined by a gravimetric method as described in AOAC–925.10. Crushed biscuits (n=4) were weighed accurately (~1 g) into a test tube and they were dried until constant weight in an oven at 105ºC. Results were expressed as percentage (%).

Thickness

After cooling, the thickness of a half of four biscuits from each set of samples (n=8) was measured using a calliper. The results were expressed in cm.

Texture (breaking force)

The texture measurements for biscuit hardness were performed using a Texture Analyser (TA–TXPlus Texture analyser, Texture Technologies Corporation, USA) equipped with a 50 kg load cell, a probe (Warner–Bratzcer, HDP / BSK knife model) with a speed at 1 mm/s and a distance prolongation of 10 mm. The force at the first major drop in force–deformation curve (Fmax) and deformation at maximum force were obtained for 4 replicates per sample. The results of hardness are expressed as N (Newton).

Colour

The colour parameters were expressed according to CIE L*a*b* scale [CIE Colorimetric Committee, 1974; McLaren

TABLE 1. Biscuit formulations: A, sucrose; B, maltitol; C, 15% stevia; D, 30% stevia; E, 60% stevia; F, 100% stevia; G, 100% stevia and coffee silverskin (CS) extract; H, 100% stevia and CS extract plus solid residue; I, 100% stevia and coffee silverskin (1.33 g); and J, 100% stevia and coffee silverskin (3.33 g).

Ingredients (g)	Biscuit formulations									
	A	B	C	D	E	F	G	H	I	J
Wheat flour	56.00	54.27	57.28	58.63	61.51	65.84	65.84	65.03	64.96	60.77
Water	20.00	19.38	20.46	20.94	21.97	23.51	0	0	23.20	24.70
Sunflower oil	7.75	7.51	7.93	8.11	8.51	9.11	9.11	9.00	8.99	9.57
Baking powder	0.55	0.53	0.56	0.58	0.60	0.65	0.65	0.64	0.64	0.68
Salt	0.37	0.36	0.38	0.39	0.41	0.43	0.43	0.43	0.43	0.46
Lecithin	0.33	0.32	0.34	0.35	0.36	0.39	0.39	0.38	0.38	0.41
Sucrose	15.00	0	13.04	10.99	6.59	0	0	0	0	0
Maltitol	0	17.62	0	0	0	0	0	0	0	0
Stevia	0	0	0.01	0.02	0.04	0.07	0.07	0.07	0.07	0.07
Coffee silverskin	0	0	0	0	0	0	0	0	1.33	3.33
Extract	0	0	0	0	0	0	23.51	23.23	0	0
Solid residue	0	0	0	0	0	0	0	1.22	0	0
TOTAL	100	100	100	100	100	100	100	100	100	100

& Riggs, 1976]. The measurements were made using a HunterLab Spectrophotometer CM–3500D colorimeter (Hunter Associated laboratory, USA). Four independent measurements of a*(redness), b*(yellowness) and L*(lightness) parameters, were carried out on different areas of the biscuit (top and bottom). The impact of the baking process on the colour of the biscuits was estimated as ΔE =Ebiscuit – Edough. E index was calculated according to the equation: $E = (L^2 + a^2 + b^2)^{1/2}$.

Processing chemical contaminants

Acrylamide

The presence of ACR was determined by liquid chromatography coupled with tandem mass spectrometry and quantified by isotopic dilution, based on the method of Arribas–Lorenzo *et al.* [2009]. The ACR was quantified using a linear calibration with standard solutions (0.1 to 100 g/L) of ACR standard containing same concentration of labelled ACR. The ACR content in the samples (n=4) was expressed as μg/kg dry weight.

Hydroxymethylfurfural

The HMF was determined by liquid chromatography coupled with a diode array detector as described by Rufián–Henares *et al.* [2006]. Sample preparation was performed in duplicate. The results were expressed as mg of HMF/kg dry weight.

Bioaccesibility

Preparation of in vitro digests

Abiotic digestion *in vitro* was carried out on three stages (oral, gastric and intestinal) as described by Hollebeeck *et al.* [2013]. Briefly, the salivary step was performed at pH 6.9, 37ºC, 5 min of incubation, 3.9 units alpha–amylase/mL under aerobic conditions. The gastric step was carried out at pH 2, 37ºC 90 min of incubation, 71.2 units pepsin/mL under anaerobic conditions while the abiotic duodenal step was run at pH 7, 37ºC 150 min, 9.2 mg pancreatin and 55.2 mg bile extract/mL under anaerobic conditions. The final mixture was centrifuged at 5000 r.p.m. for 40 min at 4ºC. The soluble fraction obtained by centrifugation was frozen at – 20ºC and freeze–dried. The resulting powder was stored at – 20ºC until analysis.

Controls containing BSA, starch and sunflower oil and wheat flour were also hydrolysed. This process was performed in duplicate. Subsequent analyses to characterise the digests, were performed in triplicate.

Glycaemic sugars

Glucose, mannose and fructose were determined using the Megazyme kit according to the manufacturer's instructions (Megazyme K–MANGL 04/13, Ireland) adapted to micro–method format. A BioTek Power WaveTM XS microplate reader (BioTek Instruments, USA) was used to measure the samples absorbance at 340 nm. D–Mannose, D–glucose, and D–fructose were used as standards at a concentration range of 0.15–0.4 mg/mL. The concentration of D–glucose, D–mannose, and D–fructose in each sample was expressed as g glucose, mannose and fructose/kg digest.

Water soluble proteins

A Bio–Rad Protein Assay, based on the method of Bradford in micro–method format was used to determine the protein concentration. All reagents were prepared according to the manufacturer's instructions (Bio–Rad Laboratories, SIG 093094). A sample blank and a reagent blank were also analysed. A calibration curve was constructed using BSA (0.05–0.5 mg/mL). The results were expressed as mg BSA/kg digest.

Free amino groups

An OPA assay was employed to determine the content of free amino groups [Michalska *et al.*, 2008]. Quantitative analysis was performed by the external standard method, using a calibration curve of Nα–acetyl–l–lysine which ranged from 10 to 250 μmol/L. The results were expressed as mol lysine/kg digest.

Chlorogenic acid

This procedure was carried out using capillary electrophoresis, according to the method of del Castillo *et al.* [2002]. The level of chlorogenic acid in the samples was quantified using a calibration curve in the range of 0.15–2.5 mmol/L chlorogenic acid. The results were expressed as μg CGA/ kg digest.

Overall antioxidant capacity

An ABTS decolourisation assay was performed according to the method of Oki *et al.* [2006]. A Trolox calibration (0.15–2 mmol) was used to calculate the overall antioxidant capacity. The results were expressed as mol Trolox/kg digest.

Statistical analysis

Data were expressed as the mean ± standard deviation. The T–test (independent samples, 2 groups) was applied to determine the differences between means (F test of Snedecor–Fisher). For the comparison of multiple means (cluster analysis) an analysis of variance (one–way ANOVA) and multiple Bonferroni test were applied. Differences were considered to be statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Quality attributes

The quality attributes of the biscuits for each of the formulations are shown in Table 2.

Moisture

No significant differences ($p>0.05$) in the moisture levels were found between the biscuits made with sucrose (6.88±0.2) and maltitol (7.54±1.7) (Table 2). Laguna *et al.* [2013] found similar results when using sucrose and maltitol in short dough biscuits.

Substituting 100% of the sucrose with stevia significantly ($p<0.05$) increased the percentage moisture of the biscuits (Table 2). These results agree with those obtained by Abdel–Shafi *et al.* [2011]. The authors have associated the increasing levels of moisture observed in the higher levels of sugar substitution with a higher gluten development and water retention in the dough. Higher moisture content will make the biscuits softer and as a consequence lower texture acceptability by consumers. In addition, this factor may also affect the shelf–life of the product.

The moisture content of the stevia biscuits was significantly reduced ($p<0.05$) by adding coffee silverskin to the formulation. No significant differences ($p>0.05$) in the moisture content were found between biscuits made with sucrose (A), maltitol (B) and stevia plus coffee silverskin (G, H, I). Addition of coffee silverskin may enhance the quality of dietary biscuits made with stevia.

Thickness

Sugar substitution (>60%) and the addition of coffee silverskin had significant ($p<0.05$) effects on the thickness of the biscuits (Table 2). The biscuits made with sucrose (A) were the thickest. Eliminating sucrose from the biscuit formulation caused a significant reduction ($p<0.05$) in the thickness.

No significant differences ($p>0.05$) were detected between the thickness of the maltitol (B) and stevia biscuits (C, E, F, H, I, J). The thinnest biscuits were those which were prepared using stevia and coffee silverskin in their formulation (G–J). The values recorded for these biscuits were significantly lower ($p<0.05$) than those corresponding to 100% sucrose (A). These results may be explained by containing less air inside the dough, or a reduction in the capacity of the dough to retain air of the biscuits made with maltitol, stevia and coffee silverskin, when compared with sucrose biscuits. These results concur with those reported by Martinez–Cervera *et al.* [2014], who found that muffins prepared using sucrose were significantly higher than those prepared using maltitol.

Texture

Table 2 shows data for the hardness of the different biscuits. The use of maltitol as a sugar replacement did not significantly affect the hardness of the biscuits. Similar results were found by Laguna *et al.* [2013] who explained this finding on the basis of the similar solubility of maltitol and sucrose in water.

The results also show a trend similar to that observed by Abdel–Shafi *et al.* [2011]. The use of 15% (C) and 60% (E) of stevia to replace sucrose, significantly decreased the breaking properties of the biscuits. The breaking force of the biscuits prepared using sucrose (A) or 100% stevia (F) did not differ significantly. Sucrose causes the formation of a weak gluten network and disperses proteins and starch, which makes the biscuit fragile [Laguna *et al.*, 2013]. The elimination of sucrose and the addition of coffee silverskin in our formulations did not significantly affect ($p>0.05$) the breaking properties of the biscuits. This suggests that in terms of texture and crispness, stevia is as suitable as maltitol to be used as a sucrose replacer.

Colour

The sucrose (A) and maltitol (B) biscuits showed similar L* and b* values indicating the usefulness of this polyol as a sucrose replacer. The substitution of sucrose by 100% stevia (F) significantly reduced ($p<0.05$) the three parameters (Table 2). Decreased a* and b* values indicate the absence of the typical brown colour characterised by the biscuits made with sucrose. This might make the stevia biscuits unacceptable to consumers.

The addition of coffee silverskin extract to the biscuits enhanced the colour profile of the stevia biscuits. The values for a* and b* of the stevia biscuits with coffee silverskin extract (G) did not differ significantly ($p>0.05$) from those made with sucrose (A). This finding supports the validity of using coffee silverskin extract as a natural colouring. The addition of coffee dietary fibre to the biscuits also affected their colour. The values for L* agree with those obtained by Popov–Raljic *et al.* [2013] for dietary biscuits. The lowest L* value found

TABLE 2. Quality properties of biscuit samples: A, sucrose; B, maltitol; C, 15% stevia; D, 30% stevia; E, 60% stevia; F, 100% stevia; G, 100% stevia and coffee silverskin (CS) extract; H, 100% stevia and CS extract plus solid residue; I, 100% stevia and coffee silverskin (1.33 g); and J, 100% stevia and coffee silverskin (3.33 g).

Biscuits	Moisture (%)	Thickness (cm)	Hardness (N)	Colour		
				L*	a*	b*
A	6.88 ± 0.22^{a}	1.11 ± 0.11^{f}	200.05 ± 66.04^{b}	72.13 ± 1.99^{f}	$4.00 \pm 1.12^{d,e}$	$23.97 \pm 1.08^{e,f}$
B	7.54 ± 1.75^{a}	$0.84 \pm 0.05^{b,c,d}$	$146.39 \pm 42.31^{a,b}$	71.94 ± 1.86^{f}	3.15 ± 0.77^{b}	24.10 ± 1.53^{f}
C	6.70 ± 0.01^{a}	$0.98 \pm 0.09^{d,e,f}$	102.26 ± 37.39^{a}	$71.40 \pm 1.32^{e,f}$	$3.86 \pm 0.95^{c,d,e}$	$23.37 \pm 1.18^{e,f}$
D	$8.83 \pm 0.01^{a,b}$	1.06 ± 0.15^{f}	204.8 ± 52.28^{b}	69.98 ± 2.01^{e}	3.06 ± 0.78^{b}	$22.16 \pm 1.47^{d,e}$
E	6.91 ± 0.09^{a}	$0.77 \pm 0.05^{a,b,c}$	112.81 ± 18.46^{a}	$71.07 \pm 1.05^{e,f}$	$3.28 \pm 0.57^{b,c}$	$23.31 \pm 0.88^{e,f}$
F	10.55 ± 0.12^{b}	$0.89 \pm 0.10^{c,e}$	$121.33 \pm 10.82^{a,b}$	66.93 ± 1.41^{d}	2.31 ± 0.55^{a}	$20.80 \pm 0.97^{c,d}$
G	7.26 ± 0.03^{a}	0.70 ± 0.00^{a}	$131.92 \pm 11.78^{a,b}$	$65.87 \pm 1.32^{c,d}$	4.09 ± 0.46^{e}	$22.36 \pm 1.08^{d,e}$
H	7.75 ± 0.10^{a}	$0.74 \pm 0.05^{a,b}$	$139.25 \pm 11.94^{a,b}$	$64.41 \pm 2.14^{b,c}$	$3.22 \pm 0.69^{b,c}$	$20.09 \pm 1.43^{b,c}$
I	7.16 ± 0.03^{a}	$0.74 \pm 0.05^{a,b}$	$129.16 \pm 10.50^{a,b}$	63.56 ± 1.39^{b}	$3.20 \pm 0.44^{b,c}$	$18.64 \pm 0.89^{a,b}$
J	$8.61 \pm 0.01^{a,b}$	$0.75 \pm 0.04^{a,b,c}$	$130.49 \pm 10.36^{a,b}$	61.70 ± 1.24^{a}	$3.30 \pm 0.25b^{c,d}$	16.89 ± 0.8^{a}

Each value represents the mean ± standard deviation. The different letters denote significant differences ($p<0.05$) between samples.

in this study (J) is of the same order of magnitude as that reported for biscuits formulated using inulin and oligofructose as a source of fibre, which are very acceptable to consumers. The parameters a* and b* may be further improved by changing the concentration of coffee silverskin extract or adjusting the baking conditions.

Figure 1 shows the appearance and development of the colour throughout the baking process (ΔE) of sucrose, maltitol, stevia and stevia with coffee silverskin biscuits. The colour differences can be categorised as: imperceptible differences (0–0.5), slight differences (0.5–1.5), just noticeable differences (1.5–3.0), marked differences (3.0–6.0), extremely marked dif-

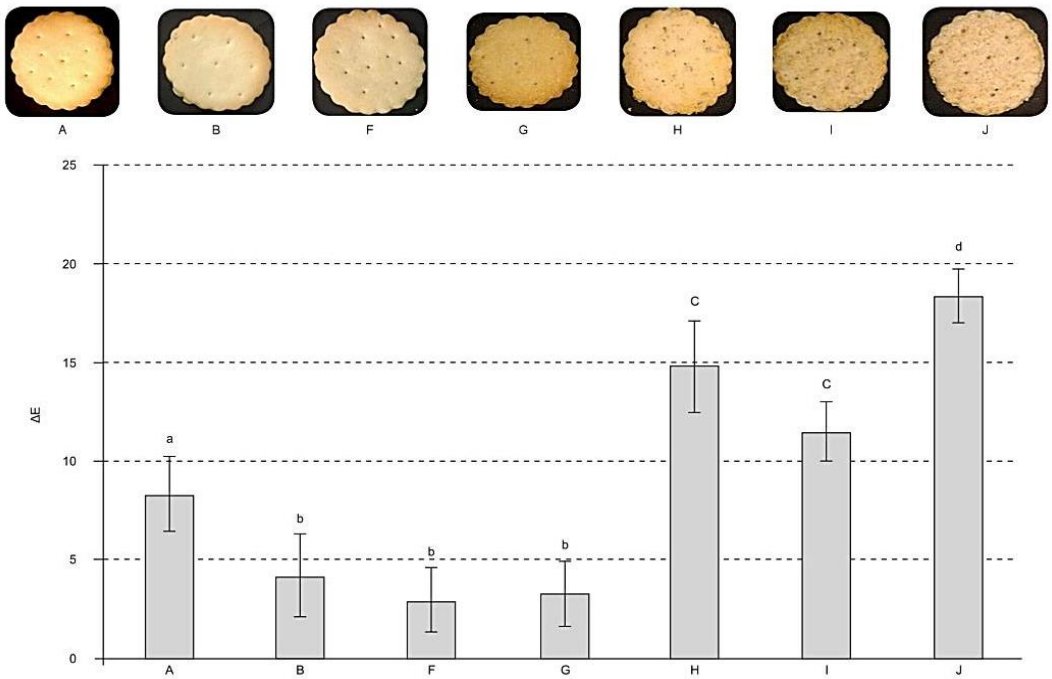

FIGURE 1. Appearance of biscuits and changes in dough colour due to baking. Changes in colour signify the difference between the biscuit colour and the dough colour (ΔE). A, sucrose; B, maltitol; F, 100% stevia; G, 100% stevia and coffee silverskin (CS) extract; H, 100% stevia and CS extract plus solid residue; I, 100% stevia and coffee silverskin (1.33 g); and J, 100% stevia and coffee silverskin (3.33 g). Bars represent the mean values and the error bars denote standard deviations. The different letters indicate significant differences ($p<0.05$) between the means of the samples.

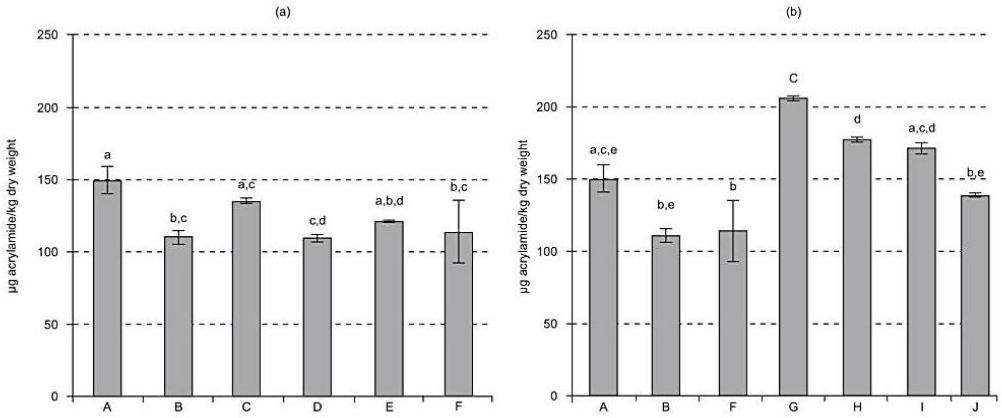

FIGURE 2. ACR content of the biscuit samples, expressed as μg acrylamide/kg dry weight. (2a) A, sucrose; B, maltitol; C, 15% stevia; D, 30% stevia; E, 60% stevia; F, 100% stevia. (2b) A; B; F; G, 100% stevia and coffee silverskin (CS) extract; H, 100% stevia and CS extract plus solid residue; I, 100% stevia and coffee silverskin (1.33 g); and J, 100% stevia and coffee silverskin (3.33 g). Bars represent the mean values and the error bars the standard deviation. The different letters indicate significant differences ($p<0.05$) between the means of the samples.

ferences (6.0–12.0) and colours of different shades (above 12.0) [Popov–Raljic *et al.,* 2013]. The baking process caused noticeable differences in the colour of the maltitol, stevia, and stevia with coffee silverskin extract biscuits. It caused extremely marked differences in the sucrose biscuits and a completely different colour or different shade in those with coffee silverskin fibre.

The substitution of sugar and the addition of solid ingredients, rich in dietary fibre, significantly affected ($p<0.05$) the colour of the biscuits (Figure 2). The poor colour development of sucrose free biscuits can be explained by a reduction in the progress of non–enzymatic browning reactions during baking. The stevia biscuits and those corresponding to the biscuits with coffee silverskin extract showed no significant differences ($p>0.05$) in colour development (ΔE). All the results (Table 2 and Figure 1) regarding the colour of the biscuits containing coffee silverskin extract support its feasibility as a natural colouring which will provide the typical golden colour expected of this type of baked products. In addition, stevia biscuits containing coffee silverskin presented an increase of the thermal impact (ΔE) compared with the sugar, maltitol and stevia formulation. The constituents of the coffee dietary fibre seem to have the biggest influence on the development of colour during baking. The coffee silverskin added to the biscuits contains 67.12% dietary fibre. Biscuits with coffee by–products usually contain up to 3.1% of dietary fibre. Recently Popov–Raljic *et al.* [2013] studied the sensory and colour properties of dietary biscuits prepared using different sources of fibre. The authors found that the fibre present in the dough of dietary biscuits has an impact on their rheological properties, water activity, viscosity and sensory characteristics, especially that of colour. According to our results, used in combination of stevia, coffee extract and coffee silverskin dietary fibre enables the production of dietary biscuits with acceptable quality properties. Coffee silverskin is an excellent natural source of dietary fibre because it contains both soluble and insoluble fibre (6.6% and 60.5%, respectively).

Processing chemical contaminants

Acrylamide

Figure 2 shows the acrylamide content of the biscuits. Sucrose and maltitol biscuits produced values of 149.89±8.96 and 110.29±4.39 μg/kg dry weight, respectively. A comparison of the sucrose biscuits with the maltitol formulation showed a significant ($p<0.05$) acrylamide mitigation (26.4%). In addition, the ACR content was significantly ($p<0.05$) reduced by substituting 30% and 100% sucrose with stevia. These results are in agreement with those obtained by Abdel–Shafi *et al.* [2011].

The addition of coffee silverskin did not inhibit ACR formation. The ACR content of the sucrose biscuits and those containing solid silverskin ingredients was not significantly different ($p>0.05$). Biscuits with coffee silverskin extract had an ACR content of 205.93±0.63 μg/kg dry weight which was significantly higher ($p<0.05$) than that found in the sucrose biscuits. Coffee silverskin extract contained 11.42 μg/L ACR which is approximately 10 times lower than that reported in coffee beverages. The FDA gives values for ACR in coffees of 175–263 μg/L [FDA, www.fda.gov]. The European Commission recommends indicative ACR values of 450 μg/kg for roast coffee (dry) and 900 μg/kg for instant (soluble) coffee (Commission Recommendation 2013/647/EU).

Rufián–Henares *et al.* [2007] reported values of ACR in commercial biscuits of 423 μg/kg which is in line with the indicative ACR value recommended by the EU for biscuits (500 μg/kg) (Commission Recommendation 2013/647/EU). The highest ACR values detected in our biscuits were 59% lower than the EU indicative values for biscuits.

Hydroxymethylfurfural

The HMF value of the sucrose formulation was significantly ($p<0.05$) higher (6.51±0.71 mg/kg dry weight) than that of the maltitol biscuits (0.83±0.07 mg/kg dry weight).

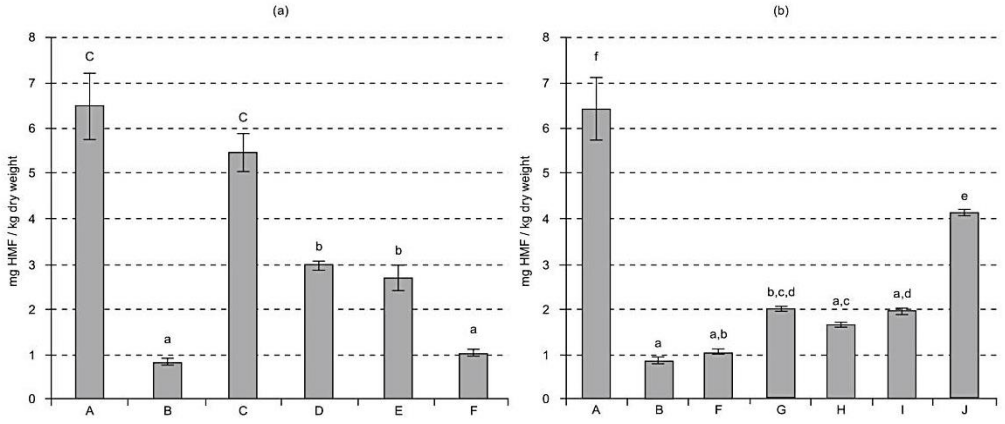

FIGURE 3. HMF content of the biscuit samples, expressed as mg HMF/kg dry weight. (3a) A, sucrose; B, maltitol; C, 15% stevia; D, 30% stevia; E, 60% stevia; F, 100% stevia. (3b) A; B; F; G, 100% stevia and coffee silverskin (CS) extract; H, 100% stevia and CS extract plus solid residue; I, 100% stevia and coffee silverskin (1.33 g); and J, 100% stevia and coffee silverskin (3.33 g). Bars represent the mean values and the error bars the standard deviation. The different letters indicate significant differences ($p<0.05$) between the means of the samples.

This large difference agrees with that described by Delgado–Andrade *et al.* [2009]. Replacing more than 30% sucrose with stevia significantly reduced the HMF content of the biscuits (Figure 3a). These results clearly show the effect of both maltitol and stevia on reducing HMF in these biscuits.

The biscuits with coffee silverskin (Figure 3b) produced HMF values significantly lower ($p<0.05$) than that found in the sucrose biscuits. The HMF content of the biscuits containing in their formulation coffee by–products ranged from 1.65±0.05 to 4.18±0.05 mg/dry weight. The highest HMF value corresponded to the biscuits that contain the highest concentration of silverskin, although this was still 35.79% lower than that found in the sucrose biscuits.

In 2005 the EFSA established a reference range for HMF in biscuits of between a minimum of 5 mg/kg and a maximum of 25 mg/kg. Delgado–Andrade *et al.* [2009] described a wide range of HMF in Spanish biscuits from 3.1 to 182.5 mg/kg with an average of 14.4 mg/kg. The combined use of stevia and coffee silverskin enables the production of biscuits with low levels of HMF (<2 mg/kg).

Bioaccesibility of food components and nutritional properties of biscuits

The chemical composition and nutritional properties of the digests of stevia biscuits (F) and those containing coffee silverksin extract plus solid residue (H) are shown in Table 3.

TABLE 3. Chemical composition and nutritional properties of biscuit digests, F (100% stevia) and H (100 % stevia with coffee silverskin extract plus solid residue).

Measurements	Digests	
	F	H
Glucose (g glucose/kg digest)	66.28±17.65[a]	56.22±3.42[a]
Fructose (g fructose/kg digest)	n.d.	n.d.
Mannose (g mannose/kg digest)	n.d.	n.d.
Total soluble proteins (mg BSA/kg digest)	129.05±8.81[a]	140.31±5.29[b]
Free amino groups (mol lysine/kg digest)	46.93±7.83[a]	40.48±3.76[a]
Acrylamide (μg acrylamide/ kg digest)	n.d.	n.d.
Chlorogenic acid (μg CGA/ kg digest)	n.d.	n.d.
Total antioxidant capacity (mol trolox/kg digest)	5.04±0.02[a]	5.07±0.02[a]

Each value represents the mean ± standard deviation. The different letters denote significant differences ($p<0.05$) between samples and n.d means non-detected.

Glycaemic sugars

The glucose content of stevia biscuit digests (66.28±17.65 g glucose/kg) was not significantly different ($p>0.05$) to that of the biscuits with coffee silverskin (56.22±3.42 g glucose/kg) (Table 3). This means that a serving of four biscuits would provide less than 3 g sucrose. The fructose and mannose content was undetectable in both digests (Table 3). Consequently, these innovative foods may be included in the category of low glycaemic foods which may be suitable for diabetics or people who want to lose weight.

Total soluble proteins

The content of soluble proteins surviving the digestion process was significantly different ($p<0.05$) (Table 3). Although most of the protein in the biscuits comes from wheat flour, coffee silverskin also contains proteins [Martinez–Saez *et al.*, 2014]. The digestibility of the proteins in flour and coffee silverskin may be different. This could explain the differences in the protein content found in the control (F) and biscuits containing coffee by–products (H).

Free amino groups

Similar levels of free amino acids were released by digestion in the control and the biscuits with coffee by–products (Table 3). According to the results of the soluble proteins and free amino acid content, coffee silverskin dietary fibre, did not appear to affect the digestibility of the flour proteins. Therefore, the addition of coffee wastes did not have a negative effect on the nutritional status of the food. Coffee silverskin fibre may be used in the formulation of dietary biscuits such as those made using apple, lemon and wheat [Bilgicli *et al.,* 2007].

Chlorogenic acid

No chlorogenic acid was detected in the digests from biscuits containing coffee by–products. This suggests that although coffee silverskin is a natural source of this compound [del Castillo *et al.,* 2013; Martinez–Saez *et al.*, 2014] it may not survive the digestion process. Vallejo *et al.* [2004] observed an 80% reduction in the total hydrocinnamic acids, including chlorogenic acid, after *in vitro* gastrointestinal digestion of broccoli.

Acrylamide

Acrylamide was not detected in the digests. In theory, acrylamide may be present at a concentration of 0.004 and 0.007 μg/mL digest in control sample (F) and biscuits containing coffee wastes (H). Berger *et al.* [2011] found a delayed acrylamide liberation and/or absorption from food compared with drinking water in the upper gastrointestinal tract indicating the influence of the food matrix on the bioavailability of acrylamide. Because acrylamide was not bioaccessible in the biscuits digests this suggests that a healthy biscuit formulation was achieved.

Total antioxidant capacity

No significant differences ($p>0.05$) were found between the digests from the control and the biscuits with coffee by–products in their formulation (Table 3). Because chlorogenic acid was not detected, the antioxidant power may be ascribed to the gluten peptides released during the digestion process [Pastoriza *et al.,* 2011].

CONCLUSIONS

The complete replacement of sucrose by stevia affected the moisture content of the biscuits. However, the addition of coffee silverskin improved this and other related physical parameters. HMF was greatly reduced and no bioaccesible acrylamide was detected in the digests of the new innovative biscuits. The nutritional value of the biscuits also improved. Our results indicate the feasibility of using coffee silverskin as a natural colouring and as source of dietary fibre, in order to achieve a healthier, nutritious, and an acceptable quality biscuit.

ACKNOWLEDGMENTS

The present research has been funded by the project with reference AGL2010–17779. This work has been funded by Naturage (AGL2010–17779), CONSOLIDER Ingenio 2010 (FUN–C–FOOD): CSD 2007–00063), ANALISYC–II, and CSIC–201370E27 projects.

Nuria Martinez–Saez would like to express her thanks for her research PhD fellowship. The authors would like to thank Mr. Diego Torres Gil for his technical assistance and for the statistical analysis of the results, and also Mr. Ignacio Rodriguez for assistance during baking. We would also like to thank Fortaleza (Vitoria) for supplying the coffee silverskin.

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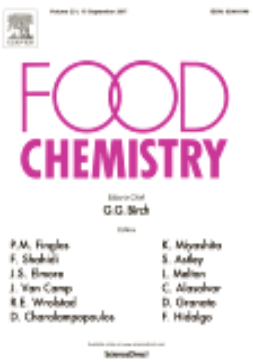
Submitted: 21 May 2014. Revised; 18 September 2014. Accepted 3 October 2014. Published on–line: 4 November 2014.

Study 2: Use of spent coffee grounds as food ingredient in bakery products

Nuria Martinez-Saez[a], Alba Tamargo García[a], Inés Domínguez Pérez[a], Miguel Rebollo-Hernanz[a], Marta Mesías[b], Francisco J. Morales[b], María A. Martín-Cabrejas[a], Maria Dolores del Castillo[a]

[a] Institute of Food Science Research (CIAL, UAM-CSIC), 28049 Madrid, Spain
[b] Institute of Food Science, Technology and Nutrition, ICTAN-CSIC, 28040 Madrid, Spain

Food Chemistry (2017), 216: 214–222
DOI: 10.1016/j.foodchem.2016.07.173



Food Chemistry 216 (2017) 114–122

Contents lists available at ScienceDirect

Food Chemistry

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Use of spent coffee grounds as food ingredient in bakery products

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[a] Institute of Food Science Research (CIAL, UAM-CSIC), 28049 Madrid, Spain
[b] Institute of Food Science, Technology and Nutrition, ICTAN-CSIC, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 31 January 2016
Received in revised form 14 July 2016
Accepted 28 July 2016
Available online 29 July 2016

Keywords:
Spent coffee grounds
Antioxidant dietary fibre
Food grade ingredient
Bakery products

ABSTRACT

The present research aimed to evaluate the use of spent coffee grounds (SCG) from instant coffee as a food ingredient and its application in bakery products. Data on physicochemical characterization, thermal stability and food safety of SCG were acquired. Evaluation of feasibility as dietary fibre was also determined. Results showed SCG are natural source of antioxidant insoluble fibre, essential amino acids, low glycaemic sugars, resistant to thermal food processing and digestion process, and totally safe. In the present work, SCG were incorporated in biscuit formulations for the first time. Low-calorie sweeteners and oligofructose were also included in the food formulations. Nutritional quality, chemical (acrylamide, hydroxymethylfurfural and advanced glycation end products) and microbiological safety and sensory tests of the biscuits were carried out. Innovative biscuits were obtained according to consumers' preferences with high nutritional and sensorial quality and potential to reduce the risk of chronic diseases such as obesity and diabetes.

1. Introduction

Spent coffee grounds (SCG) are the most abundant coffee by-product (45%) generated in coffee beverage preparation and instant coffee manufacturing (Murthy & Naidu, 2012b). About 2 kg of wet SCG are obtained from each kg of instant coffee produced, with an annual generation of around 6 million tons worldwide (Mussatto, Machado, Martins, & Teixeira, 2011). To date, several applications have been described for SCG, mainly as biofuels, composts, animal feed, biosorbents and enzymes. Recently, an increase in food and health application is occurring (Del Castillo, Fernandez-Gomez, Martinez-Saez, Iriondo, & Mesa, in press). Previous studies performed by our group indicated the importance of SCG as source of antioxidant dietary fibre (Del Castillo, Martinez-Saez, & Ullate, 2014).

Consumers are concerned about caloric content and glycaemic index (GI) of the food as well as balanced nutrition comprising dietary fibre content. The benefits of low GI diets extend beyond weight loss and have favourable effects on obesity-related diseases such as type 2 diabetes (Esfahani, Wong, Mirrahimi, Villa, & Kendall, 2011). Food industry needs to fulfil the increasing consumer's demand of healthier and tastier foods. The search for healthier and tasty food as for instance bakery products is a necessity in our population. Maillard reaction is the main chemical event occurring during coffee roasting and baking. The reaction affects nutritional quality, safety and sensory value. Maillard reaction products present health promoting (melanoidins) and potential harmful effects (acrylamide, furans and advanced glycation end products) (Tamanna & Mahmood, 2015). The present work aims to evaluate the use of SCG as food ingredient in innovative bakery products with high nutritional and sensorial quality and potential to reduce the risk of chronic diseases such as obesity and diabetes.

2. Materials and methods

2.1. Reagents

Bradford reagent was provided by Bio-Rad Laboratories S.A; α-amylase from human saliva (type IX-A), porcine pepsin from gastric mucosa (3.200–4.500 U/mg protein), pancreatin from porcine pancreas, porcine bile extract, bovine serum albumin (BSA), chlorogenic acid (CGA) (3-CGA), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS)), Folin-Ciocalteu reagent, Nα-Acetyl-L-lysine, ortho-phthalaldehyde (OPA), 1-deoxy-1-morpholinofructose (DMF), nitroblue tetrazolium (NBT), butylated hydroxytoluene and proteinase k from *Pichia pastoris* were from Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent was provided by Bio-Rad Laboratories S.A, glucose kit from Spinreact (Gerona,

* Corresponding author.
E-mail address: mdolores.delcastillo@csic.es (M.D. del Castillo).
URL: http://www.cial.uam-csic.es (M.D. del Castillo).

http://dx.doi.org/10.1016/j.foodchem.2016.07.173
0308-8146/

Spain), total dietary fibre kit from Megazyme (International Ireland Ltd) and advanced glycation end products assay kit from Lamider® (México, D.F., México). Buffered peptone water (BPW) from Biocult, plate count agar (PCA) medium from BD Diftco™, brain heart infusion (BHI) agar from BD Bacto™ (New Jersey, USA) and sabouraud dextrose agar (SDA) with chloramphenicol from CONDA (Pronadisa). Water was purified using Milli-Q and Elix system. All other chemicals and reagents were of analytical grade.

2.2. Apparatus

BioTek powerWaveTM XS (BioTek Instruments, U.S.A) and FP-6200 (JASCO, Easton, U.S.A) microplate spectrometers, Agilent G16000A capillary electrophoresis (Agilent, Madrid, Spain), convection oven (Romag S.A, Barcelona, Spain), UN 500 universal oven (Memmert, Germany), Telstar Lyobeta-15 lyophilizer (Telstar, Spain), CertoCLAV A-4050 autoclave (CertoCLAV, Austria), AW Sprint TH-500 water activity system (Novasina, Switzerland), Shimadzu HPLC system (Kyoto, Japan), Agilent 1200 liquid chromatograph coupled to an Agilent Triple Quadrupole MS detector (Agilent Technologies, Palo Alto, CA, USA) and Biochrom 30 Amino Acid Analyzer (Cambridge, UK) were used for analysis. Stomacher® 400 Circulator (Seward, U.K.), horizontal laminar flow bench Mini-H (Telstar, Spain), Nüve EN120 incubator (Nüve, Turkey) and SANYO Mir 154 incubator (SANYO Electric Biomedical Co., Ltd. UK) were also needed for microbiological studies.

2.3. SCG samples

Raw coffee by-product: SCG from industrial soluble coffee production of the Robusta specie were provided by Prosol S.A (Spain) and stored under −20 °C until preparation of the biscuits and microbiological analysis. Information regarding to the thermal stability of SCG dietary fibre was obtained by treating the sample under those conditions used for the baking of biscuits (185 °C, 16 min).

Stabilized SCG: Different drying conditions were applied on raw SCG (40 °C, 70 °C and 100 °C) until constant weight was achieved. Samples were stored at room temperature in dry and fresh place for 24 h, until analyses of their physical properties (moisture loss, moisture content and water activity) and microbiological quality. Energy consumption (kW·h) was calculated for each drying condition by correlation of consumption and operating temperature. Calculation was performed in order to validate the feasibility of the method for its industrial use.

Freeze-dried SCG: The raw material was lyophilized and stored in dry and cool place until analysis of its physico-chemical properties (moisture, water activity, ashes, dietary fibre, proteins, amino acid composition, fat, carbohydrates and antioxidant capacity) and safety by means of the quantification of food processing contaminants (acrylamide and hydroxymethylfurfural).

In vitro oral gastrointestinal digestion was carried out to evaluate the resistance of SCG to digestion process. The freeze-dried SCG were digested as described by Hollebeeck, Borlon, Schneider, Larondelle, and Rogez (2013), with slight modifications. All three stages (salivary, gastric and duodenal) were performed in the same flask covered with aluminium foil. Approximately 1.2 g of SGC were weighed. Conditions were set up as follows: salivary step (pH 6.9, 10 ml, 5 min, 3.9 U α-amylase/ml, aerobic), gastric step (pH 2, 13 ml, 90 min, 71.2 U pepsin/ml, aerobic), and abiotic duodenal step (pH 7, 16 ml, 150 min, 9.2 mg pancreatin and 55.2 mg bile extract/ml, aerobic). The obtained digests were centrifuged and separated in two fractions, supernatant and precipitate. The content of dietary fibre was determined in the precipitate (non-digestible fraction also called colonic fraction). Finally, the soluble fraction was treated to mimic human intestinal reabsorption of bile salts. Cholestyramine resin (10% w/v) previously activated was used as bile acids precipitating agent (Edwards & Slater, 2009) for 1 h at room temperature by mixing with magnetic stirrer. Cholestyramine was removed by centrifugation and gravimetric filtration. The antioxidant capacity and total phenol content of supernatants free of bile salts containing the bioaccesible compounds in the intestinal lumen were analysed. A food control composed by starch, bovine serum albumin and oil was included. Digestion process was carried out in duplicate and the analysis of the samples in triplicate.

Aqueous SCG extracts: An extractable fraction from freeze-dried SCG was obtained with hot water (50 mg/ml) at 100 °C for 10 min as described in the patent WO 2013/004873. Extracts were stored at −20 °C until use. Further chemical analyses (free glucose, antioxidant capacity, total phenolic content, CGA and caffeine) were carried out to gain insight on soluble compounds present in SCG. The analysis of the fraction aimed to assess the interest for extracting other compounds beside dietary fibre from the brewing coffee by-products and to reinforce its value as natural abundant source of antioxidant dietary fibre.

2.4. Biscuits samples

2.4.1. Food ingredients

For the biscuits formulations, all basic ingredients were purchased at specialized and certified food markets. Food grade soy lecithin was provided by Manuel Riesgo S.A. (Spain) and maltitol was supplied by a national food company. The commercial stevia sweetener powder which contained 3% steviol glycosides, was supplied by Gerblé (Spain) and oligofructose powder (ORAFTI®P95) by Beneo-Orafti.

2.4.2. Biscuits formulations

A total of 6 innovative free-sugar biscuits (B1, B2, B3, B4, B5, B6) were formulated as indicated in Table 1. Biscuits were prepared using as basic ingredients wheat flour and sunflower oil. Egg was not incorporated and sucrose was replaced by stevia and/or maltitol as natural hypocaloric sweeteners. SCG were included as antioxidant insoluble dietary fibre. The amount of SCG added to the biscuits ranged 3.5–4.4%, in order to achieve the nutritional claims "source of fibre" (⩾3 g fibre/100 g biscuit) and "high fibre content" (⩾6 g fibre/100 g biscuit). Oligofructose was included as

Table 1
Innovative biscuit formulations: B1 – 100% stevia (ST) and spent coffee grounds (SCG); B2 – 100% ST. oligofructose (OF) and SCG; B3 – 30% ST. 70% maltitol (MT) and SCG; B4 – 30% ST. 70% MT. OF and SCG; B5 – 100% MT and SCG; B6 – 100% MT. OF and SCG.

Ingredients (g)	B1	B2	B3	B4	B5	B6
Wheat flour	61.54	59.37	55.11	55.53	52.83	53.35
Water	21.98	21.20	19.68	18.21	18.87	17.49
Sunflower oil	8.52	8.22	7.63	7.06	7.31	6.78
Baking powder	0.60	0.58	0.54	0.50	0.52	0.48
Salt	0.41	0.39	0.36	0.34	0.35	0.32
Soy lecithin	0.36	0.35	0.32	0.30	0.31	0.29
MT	0.00	0.00	11.81	10.83	16.04	14.87
ST	2.20	2.12	0.60	0.56	0.00	0.00
OF	0.00	3.53	0.00	3.03	0.00	2.91
SCG	4.40	4.24	3.94	3.64	3.77	3.50
Total	100	100	100	100	100	100
Estimated calories (kcal/100 g biscuit)	407	390	346	335	325	316
Estimated fibre content (g fibre/100 g biscuit)	3.6*	7.5†	3.1*	6.4†	3.0*	6.1†

* B1. B3 and B5 might be "source of fibre" (⩾3 g fibre/100 g biscuit).
† B2. B4 and B6 might be "high fibre content" (⩾6 g fibre/100 g biscuit).

enhancer of glucose tolerance (soluble fibre), gut microbiota (prebiotic), texture and taste. The preparation of the doughs follows the same procedure performed by Garcia-Serna, Martinez-Saez, Mesias, Morales, and Del Castillo (2014). The biscuits were baked at 185 °C for 16 min in an air recirculation oven.

Three commercial biscuits with high consumer adhesion were purchased and used as references in the present research. The biscuit containing maltitol and isomaltitol as sweeteners and 3.6% dietary fibre was named as C1. C2 biscuit was based on maltitol as sweetener and 8.3% dietary fibre. C1 and C2 were used as references in the sensorial analysis since those contain similar composition to that of the innovative foods. The commercial biscuit (CSB) containing sucrose as sweetener was used as a control of the average formation of potential harmful compounds during food processing (see ingredients and nutritional composition provided in the Supplementary material section of the article).

Proteins, soluble free amino groups, fructosamine, advanced glycation end products in the new food (B2) and commercial sucrose biscuit (CSB) were evaluated. Microbiological quality and food processing contaminants were also analysed on B2 biscuit and a control biscuit elaborated using the same ingredients and recipe employed for preparing B2 without addition of SCG. A sensorial test was carried out to evaluate the sensorial quality of all the innovative biscuits (B1–B6).

B2 was selected as new healthier food due to its high nutritional and sensorial quality and safety. A limited formation of potential harmful Maillard reaction products (MRPs) was expected by replacing sugar sweetener by stevia. This sweetener presents benefits for diabetes.

2.5. Physicochemical analyses

2.5.1. Moisture

Moisture content was determined by gravimetric method as described in AOAC–925.10, at 105 °C until constant weight. Results were expressed as%.

2.5.2. Water activity (a_w)

It was measured in a water activity system at 25 °C. The calibration was carried out as indicated by the manufacturer. A humidity standard, SALT-T75, was used for the basic calibration. This standard was inserted in the measuring chamber and the instrument carried out the calibration whilst at the same time determining the heating compensation value.

2.5.3. Ashes

The amount of ashes was quantified by the AOAC-923.03 based on the complete incineration of organic matter at 550 °C. Results were expressed as%.

2.5.4. Dietary fibre

Insoluble (IDF), soluble (SDF) and total (TDF) dietary fibre were determined by using enzymatic-gravimetric assay based on the AOAC-991.43 and AACC-32.07.01 method. Results were expressed as%.

2.5.5. Total protein

Content of proteins was determined by Kjeldahl mineralization followed by a colorimetric analysis of nitrogen for quantification (AOAC-32.1.22, 920.87). NH_4Cl was used for standard calibration curve. A conversion factor (6.25) was used to calculate proteins content. Results were expressed as % dry matter (d.m.).

2.5.6. Soluble proteins

Bio-Rad Protein Assay (Bio-Rad Laboratories) based on the method of Bradford was used in micro-method format to determine protein concentration. Briefly, a solution of Bradford reagent (1:4, reagent:milli-Q water) was prepared and filtered using Whatman 4 filter. Ten μl of sample and 200 μl of Bradford solution were placed in a multi-well microplate. Samples were incubated for 5 min at room temperature, and the absorbance was measured at 595 nm. Sample blank and reagent blank were also analysed. A calibration curve was constructed using BSA (0.05–0.5 mg/ml). All measurements were performed in triplicate. Results were expressed as mg BSA/g.

2.5.7. Free amino groups

An OPA assay was employed to determine the content of free amino groups (Michalska, Amigo-Benavent, Zielinski, & del Castillo, 2008). Quantitative analysis was performed by the external standard method, using a calibration curve of Nα-acetyl-L-lysine (10–250 μmol/l). The results were expressed as mg Lys eq./g biscuit and mg Lys eq./g protein.

2.5.8. Amino acids composition

Amino acid analysis was performed according to AOAC-994.12 method, which is based on acid hydrolysis of sample followed by HPLC with post column derivatization using ninhydrin. Analysis in triplicate was performed and results were expressed as % protein.

2.5.9. Fructosamine

Prior to the analysis the biscuits were treated with PBS (1 g sample in 10 ml) containing BHT (0.2 mM) for 48 h at 4 °C. Later, enzymatic hydrolysis was performed by incubating sample overnight with proteinase k (0.2% p/p) at 37 °C. The reaction was stopped at 65 °C for 1 h and centrifuged (1000 rpm, 10 min) to keep supernatant for further analysis.

NBT assay was performed to determine fructosamine following the micromethod of Vlassopoulos, Lean, and Combet (2013). Briefly, samples (25 μl) were added to sodium carbonate buffer (100 μl, 100 mM, pH 10.8) with nitroblue tetrazolium (0.25 mM). Microplates were incubated for 20 min at 37 °C and measured spectrophotometrically against control at 530 nm. The fructosamine analogue DMF (1-deoxy-1-morpholinofructose) was used as standard. All measurements were performed in triplicate and expressed as μg DMF eq./100 g biscuit and μg DMF eq./mg protein.

2.5.10. Fat

Total fat content was quantified by Soxhlet extraction with petroleum ether (AOAC 945.16) applying previously an acid hydrolysis (AOAC 922.06). Results were expressed as % d.m.

2.5.11. Total carbohydrates

Carbohydrates were calculated by difference following this formula:

100 – (weight in grams [protein + fat + water + ash + fibre])

Results were expressed as % d.m.

2.5.12. Free glucose

The procedure for determining the amount of glucose was performed by using an enzymatic kit as per the manufacturer's instructions. Analysis was carried out in triplicate. Results were expressed as mg/100 g SCG.

2.5.13. Caffeine and CGA

The procedure was performed according to Del Castillo, Ames, and Gordon (2002). Separation was carried out in a capillary electrophoresis system with an ultraviolet–visible detection. Calibration curves of caffeine (0.25–5.15 mmol/l) and CGA

(0.15–2.5 mmol/l) were constructed. The analyses were performed in triplicate. Results were expressed as% 3-CGA (w/w) and % caffeine (w/w).

2.5.14. Total phenolic content

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method as described by Contini, Baccelloni, Massantini, and Anelli (2008) adapted to a micro-method format. CGA calibration curve (0.1–0.8 mg/ml) was used for quantification. Measurements were performed in triplicate and results were expressed as % CGA equivalent (eq.) (w/w).

2.5.15. Overall antioxidant capacity

Direct $ABTS^{\cdot+}$ assay or QUENCHER assay, was carried out according to Açar, Gökmen, Pellegrini, and Fogliano (2009). Ten mg raw SCG were mixed with 90 mg cellulose and stirred, 10 mg of the mixture were mixed together with 1.7 ml of $ABTS^{\cdot+}$ solution in a thermomixer (25 °C, 2 min, 600 rpm). After centrifugation, absorbance of the supernatant was measured in microplate. A CGA and Trolox calibration curve (10–200 μg/ml) was used. Measurements were performed in triplicate and results expressed as % Trolox eq. (w/w) and % CGA eq. (w/w).

Indirect $ABTS^{\cdot+}$ decolourisation assay, in terms of radical scavenging activity was employed as described by Oki, Nagai, Yoshinaga, Nishiba, and Suda (2006). Absorbance was measured in microplate. Aqueous solutions of CGA (0.15–2.0 mmol/l) were used for calibration. All measurements were performed in triplicate and results expressed as % CGA eq. (w/w).

2.6. Food safety

2.6.1. Microbiological analyses

Count of (1) total aerobic microorganisms, (2) aerobic microorganisms forming endospores and (3) moulds and yeasts, was carried out. All assays were performed in sterile conditions and with previous solubilisation of SCG (10 g) in BPW (90 ml) by using a stomacher (230 rpm, 1 min). Different conditions were set for each analysis: (1) pour plate method, PCA medium, incubation at 30 °C 72 h; (2) pour plate, BHI agar medium, pre-incubation (80 °C, 10 min) and incubation at 37 °C 48 h; and (3) spread method, SDA with chloramphenicol and incubation at 25 °C 120 h. Results were expressed as colony forming units (CFU)/g.

2.6.2. Food processing contaminants

Content of acrylamide (ACR) was determined by liquid chromatography coupled with tandem mass spectrometry and quantified by isotopic dilution, based on the method of Mesías, Holgado, Márquez-Ruiz, and Morales (2015). For quantification a curve calibration with standard ACR (1–100 μg/l) was constructed. The limit of the quantitation was set at 16 μg/kg. The accuracy of the results were recently demonstrated for crispbread in an inter-laboratory comparison study launched by Food Analysis Performance Assessment Scheme (FAPAS) program (2015), yielding a *z*-score of 0.3. Results (n = 4) were expressed as μg ACR/kg dry weight. Hydroxymethylfurfural (HMF) was determined by liquid chromatography coupled with a diode array detector as described by Rufián-Henares, Delgado-Andrade, and Morales (2006). The limit of quantification was set at 0.6 mg/kg. Analysis was performed in duplicate. Results were expressed as mg HMF/kg dry weight.

2.6.3. Advanced glycation end products

Prior to analysis sample preparation was carried out as described in Section 2.5.9. Fluorescent and total AGEs were determined as follows:

Fluorescence of AGEs was measured at λ_{Ex} = 360 ± 40 nm and λ_{Em} = 460 ± 40 nm in a microplate fluorescence reader. Samples were analysed in triplicate. Results were expressed as fluorescence units (FU)/100 g biscuit.

Competitive enzyme-linked immunosorbent assay (ELISA) kit was employed to determine total AGEs in the biscuit samples. The assay was performed according to the manufacturer's instructions. Results were expressed as total AGEs units (U)/100 g biscuit.

2.7. Sensorial quality

Sensorial analysis of the innovative (B1, B2, B3, B4, B5, B6) and commercial (C1, C2) biscuits was carried out applying a hedonic sensory test. Sensory evaluation was performed in different sessions involving in total 26 untrained panellists. Colour, texture, taste and overall acceptance of the selected biscuits were tested. Results of the verbal scale test were converted into a 7-point scale scoring 1 (lowest)-7(highest) and the average of the panellists' scores was calculated.

2.8. Statistical analysis

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software Inc., California, USA). Data were expressed as the mean value ± standard deviation (SD). Analysis of variance (ANOVA) and the Fisher post hoc test were applied to determine differences between means. Differences were considered to be significant at $p < 0.05$.

3. Results and discussion

3.1. Validation of SCG as food ingredient

3.1.1. Physicochemical characterization

Data on physicochemical characterization are summarized in Table 2. Freeze-dried SCG presented 3.6% moisture equivalent to a_w value lower than 0.1. Growth of microorganisms and chemical degradative reactions might be widely decreased. The content in

Table 2
Physico-chemical characterization of spent coffee grounds (SCG).

Analysis	SCG
Moisture (%)	3.60
Aw	<0.10
Ashes (%)	0.50
Polysaccharides (%)	13.10
Free Glucose† (mg/100 g)	3.38
Dietary fibre	
TDF (%)	47.30
IDF (%)	41.63
SDF (%)	5.67
Total protein (%)	11.20
Nitrogen (%)	1.79
Fat (%)	24.30
ABTS	
Direct	
% Trolox eq. (w/w)	0.17
% CGA eq. (w/w)	0.33
Indirect†	
% CGA eq. (w/w)	0.39
TPC†	
% CGA eq. (w/w)	0.18
CGA†	
% 3-CGA (w/w)	0.01
Caffeine†	
% caffeine (w/w)	0.20
kcal/100 g	411

Results are expressed as mean (n = 3).
† Analyses performed on aqueous SCG extracts.

ashes (0.5%), fat (24.3%) and energetic value (411 kcal/100 g) is in concordance with those described by others authors regarding SCG from soluble coffee (Lago, Antoniassi, & Freitas, 2001; Pujol et al., 2013). As in coffee bean, carbohydrates are the main components present in SCG (Table 2). Polysaccharides, different to those quantified as dietary fibre, ranged 13.1%, and free glucose content was nearly insignificant (0.04 g/100 g SCG) indicating low presence of glycaemic sugars. Results for total dietary fibre (47.3 g/100 SCG) are in line with those reported by Lago et al. (2001) and Vardon et al. (2013). SCG dietary fibre is mainly composed of insoluble fibre (88% total dietary fibre), representing the 41.6% of spent coffee. SCG are rich in mannose, galactose, glucose and arabinose polymerized into hemicellulose and cellulose (45.3–51.5%, w/w) (Ballesteros, Teixeira, & Mussatto, 2014; Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011) and high presence of galactomannan (Simões, Nunes, Domingues, & Coimbra, 2013). Lignin is also present in a significant amount in SCG (19–26% w/w) (Ballesteros et al., 2014; Pujol et al., 2013).

Caffeine and CGA are the major bioactive compounds of coffee. Freeze-dried SCG showed values of 200 mg caffeine/100 g and 10 mg CGA/100 g SCG, respectively. CGA content ranged lower values than those described by others authors (Bravo et al., 2012; Cruz et al., 2012; Murthy & Naidu, 2012a; Mussatto et al., 2011; Panusa et al., 2013). These differences might be attributed to solvent extraction, set conditions, method of quantification, brewing method and origin of SCG. However, caffeine content perfectly fits in with those reported by Cruz et al. (2012). Regarding the overall antioxidant capacity measured by ABTS method (Table 2), 0.33% CGA eq. (w/w) was found for SCG. The contribution of phenolic compounds to overall antioxidant capacity is shown in Table 2. The TPC of SCG ranged 0.18% CGA eq. (w/w) corresponding to the 50% of total antioxidant capacity. This result was in concordance with those reported by Pujol et al. (2013) for SCG aqueous extracts from soluble coffee.

As far as protein content is concerned, SCG showed significant amount of proteins (11.2%, w/w). These values were inside the range reported by other authors, 6.7–9.9% (Lago et al., 2001) and 12.8–16.9% (Cruz et al., 2012). SCG protein content exceeds the amount presents in coffee bean due to the concentration of non-extracted components such proteins in coffee residues during instant coffee preparation.

Table 3
Amino acids composition (% protein) of spent coffee grounds (SCG).

Amino acids	SCG (% protein)
Alanine (Ala)	2.34 ± 0.71
Arginine (Arg)[a]	0.01 ± 0.01
Aspartic acid (Asp)	5.10 ± 0.71
Cysteine (Cys)	0.15 ± 0.01
Glutamic acid (Glu)	4.13 ± 0.56
Glycine (Gly)	2.68 ± 0.17
Histidine (His)[a]	0.39 ± 0.08
Isoleucine (Ileu)[a]	0.94 ± 0.13
Leucine (Leu)[a]	2.49 ± 0.37
Lysine (Lys)[a]	0.59 ± 0.10
Methionine (Met)[a]	0.26 ± 0.03
Phenylalanine (Phe)[a]	1.18 ± 0.22
Proline (Pro)	1.63 ± 0.29
Serine (Ser)	0.57 ± 0.10
Threonine (Thr)[a]	4.71 ± 1.01
Tyrosine (Tyr)	0.33 ± 0.09
Valine (Val)[a]	1.69 ± 0.14
Essential AAs (% total)	42.0 ± 1.2
BCAA (Val + Leu + Ile)	5.12 ± 0.6
AAA (Phe + Tyr)	1.51 ± 0.3
Fisher Ratio	3.40 ± 0.3

Results are expressed as mean ± standard deviation (n = 3).
[a] Essential amino acids.

Data on amino acids (AAs) content were analysed (Table 3). The essential AAs comprise 42% of the total SCG amino acids which is smaller than the 49% described by Lago et al. (2001). Glutamic acid, threonine, aspartic acid and leucine presented the upper values, unlike data previously reported for SCG (glutamic acid, leucine, valine and isoleucine) (Lago et al., 2001). Fischer's ratio is the ratio of branched-chain AAs (BCAAs: leucine, valine, isoleucine) to aromatic AAs (AAA: phenylalanine, tyrosine) and play an important role, mainly, in liver diseases (Ishikawa, 2012). As can be observed (Table 3) a value of 3.39 for the Fischer's was obtained which is higher than those described for SCG, coffee pulp and soymeal (Campos-Vega, Loarca-Piña, Vergara-Castañeda, & Oomah, 2015). Proteins with high BCAA, Fischer's ratio and low AAAs are sought for functional foods field in order to support special requirements in malnourished patients associated to cancers, burns, trauma, and liver failure among others (Oomah, 2001 and references therein).

3.1.2. Thermal stabilization of SCG

Influence of drying conditions (40 °C, 70 °C, 100 °C and lyophilisation) on moisture loss (ML), moisture content (MC) and a_w of SCG is presented in Table 4. Significant differences were not found between SCG-70, SCG-100 and SCG-Ly ($p > 0.05$) for ML (95.96–96.31%) and MC (2.3-2.51%) ($p > 0.05$), However, SCG-40 showed values significantly lower and higher ($p < 0.05$) for ML and MC, respectively. Regarding a_w parameter, the higher temperature the lower available water was observed (0.35–0.05) and the minimum value was obtained by SCG-Ly, prolonging the shelf life of the product. In contrast, lyophilisation energy consumption (99.3 kW·h) was far superior to SCG-40, SCG-70 and SCG-100 (0.163, 0.177, 0.198 kW·h). Therefore, an adequate balance comprising product stability and moderate energy consumption might lead to select as the best drying conditions, 40 °C and 70 °C.

3.1.3. Food safety of SCG

Results for raw SCG showed values below 10^2 CFU/g for endospores, 10^4 CFU/g of total aerobic microorganisms, content of yeasts lower than 10^7 CFU/g and absence of moulds. Regarding the thermal stabilized SCG, values below 10 CFU/g were found for all microorganisms under study. No microbiological regulations have been stablished for coffee. The principal hazard in terms of safety is the presence of ochratoxin A (OTA), a mycotoxin released by *Aspergillus* and related species with high incidence in coffee (Codex Alimentarius, 2009). OTA is potentially nephrotoxic, carcinogenic, teratogenic and possibly genotoxic. The Joint Expert Committee for Food Additives (JECFA) established a provisional tolerable weekly intake of 100 ng/kg of body weight and maximum permissible limits relative to 5 and 10 μg/kg in roasted ground coffee and instant coffee, respectively (European Commission Regulation (EU) No 123/2005, 2005). The absence of moulds in SCG reduces the risk of OTA contamination. Thus, SCG presented excellent microbiological quality and thereby SCG becomes a safe food ingredient.

Table 4
Physical characterization -moisture loss (ML) (%), moisture content (MC) (%) and water activity (a_w)- of SCG stabilized at 40°, 70°, 100 °C and lyophilized (SCG-40, SCG-70, SCG-100, SCG-Ly).

	ML (%)	MC (%)	a_w
SCG-40	92.76 ± 0.66[a]	4.51 ± 0.41[a]	0.35 ± 0.02[a]
SCG-70	96.31 ± 0.20[b]	2.30 ± 0.13[b]	0.14 ± 0.01[b]
SCG-100	95.97 ± 1.16[b]	2.51 ± 0.72[b]	0.11 ± 0.01[c]
SCG-Ly	95.96 ± 1.16[b]	2.52 ± 0.72[b]	<0.05[d]

Data are presented as mean ± standard deviation (n = 3). Different letters indicate significant differences ($p < 0.05$) between the samples of the same column.

Regarding food processing contaminants, two neoformed contaminants have gained much interest, ACR and HMF. Both are considered as potentially carcinogenic to humans or precursors of potentially carcinogenic compounds. SCG presented 37.2 ± 0.4 μg ACR/kg d.m. which were less than those found in roasted coffee (256 μg/kg) and instant coffee (1123 μg/kg) based on 2010 monitoring data (EFSA, 2012). Moreover, ACR values detected in our samples were 92–96% (SCG) lower than the indicative values proposed by European Commission (2013). Regarding the HMF, 61.3 ± 0.4 mg HMF/kg d.m. was found in SCG. Different authors reported HMF levels in coffee (100–1900 mg/kg) and instant coffee (400–4100 mg/kg) superior to our results (Capuano & Fogliano, 2011 and references therein). In this sense, SCG are small source of ACR and HMF.

3.1.4. Evaluation of SCG as dietary fibre

Results relative to thermal resistance showed that insoluble fibre did not experiment change after submitting SCG at 185 °C for 16 min. SCG was also resistant to *in vitro* oral gastrointestinal digestion ($p > 0.05$). Moreover, soluble fraction of SCG digests exhibited low antioxidant capacity by ABTS and TPC relative to 46.14 ± 3.61 mg CGA eq./g digest (0.06% w/w SCG) and 15.56 ± 0.95 mg CGA eq./g digest (0.019%, w/w SCG), respectively. These results indicate soluble fraction of SCG digest contribute in approximately 18% total antioxidant capacity of SCG and therefore the remaining insoluble fraction might still maintain most antioxidant capacity linked to the insoluble fibre which would be in concordance with data reported by Jiménez-Zamora, Pastoriza, and Rufián-Henares (2015).

In conclusion, it can be stated that SCG from instant coffee process are natural source of antioxidant insoluble fibre, essential AAs, with low glycaemic sugars, resistant to thermal food processing conditions and digestion process, and totally safe. Therefore, SCG could be incorporated as food ingredient in bakery products for human consumption.

3.2. Food application of SCG

3.2.1. Nutritional quality and safety of innovative biscuits

With regard to nutritional value, in terms of extractable protein content, the innovative biscuit containing SCG (4%), stevia and oligofructose (B2) (19.47 ± 2.37 mg/g biscuit) did not present significant difference ($p > 0.05$) with the commercial sucrose biscuit (CSB) (17.52 ± 1.44 mg/g biscuit). In contrast, amino acids content was significantly lower ($p < 0.05$) for CSB than for B2 biscuit, presenting values of 0.81 ± 0.03 and 1.03 ± 0.01 mg Lys eq./g biscuit (46.16 ± 2.33 and 53.3 ± 6.6 mg Lys eq./g protein), respectively.

Results concerning food safety showed values of 10^4 CFU/g for total aerobic microorganisms and endospores, and moulds and yeasts lower than 10^2 CFU/g for the innovative biscuit B2 and the biscuit elaborated using the same ingredients and recipe employed for preparing B2 without incorporation of SCG (control biscuit). These findings fit with those values stablished by the national microbiological standards for biscuits (RD 1124/82, 1982). The addition of SCG to the biscuit formulations did not increase the content of microorganisms suggesting the good microbiology quality of food ingredient.

Values of ACR and HMF in B2 were of 166 ± 0.2 μg/kg and 8.3 ± 0.1 mg/kg, respectively. Values of 169 ± 7.1 μg ACR/kg and 6.4 ± 0.3 mg HMF/kg were found in biscuit elaborated using the same ingredients as B2 without addition of SCG (control biscuit). As can be observed, no significant differences ($p < 0.05$) in ACR values were detected between the two biscuits. Therefore, results seem to indicate SCG do not contribute to the formation of ACR during baking. B2 presented an ACR content of 67% lower than those indicative ACR values (500 μg/kg biscuit) by the European Commission (2013) and within the range stablished for HMF in biscuits by the EFSA (2005) (5 mg/kg–25 mg/kg). As a consequence, the innovative biscuit can be considered safe for consumption.

On the other hand, CSB showed significant higher formation of Amadori compounds, in particular fructosamine (621.28 ± 30.9 μg DMF eq./100 g biscuit and 96 ± 5 μg DMF eq./mg protein) than B2 (498.04 ± 4.9 μg DMF eq./100 g biscuit and 68 ± 7 μg DMF eq./mg protein), suggesting that faster evolution of the early stage of Maillard reaction occurred in the commercial sucrose biscuit. At this stage of the reaction, reducing sugars react with free amino groups to form Schiff base and then produce stable ketoamines (Culetu, Fernandez-Gomez, Ullate, del Castillo, & Andlauer, 2016). The innovative biscuit (B2) does not contain reducing sugar to favour the formation of fructosamine unlike CSB. Negative consequence from Maillard reaction is the loss of nutritive value of proteins involved, with a loss of food quality. These Amadori products might reduce the bioavailability of essential amino acids such as lysine. Nevertheless, different studies have demonstrated the health-beneficial properties of Maillard reaction products (MRPs) (Tamanna & Mahmood, 2015). Nowadays, the benefit/risk ratio of the Maillard reaction products in foods and health is a matter of great interest and controversy.

AGEs formation, in the advanced step of Maillard reaction, leads to a decrease of food safety (Poulsen et al., 2013). Fluorescent AGEs were formed in significantly higher amounts ($p < 0.05$) in CSB (81.82 ± 0.0 FU/100 g) than in B2 (60.78 ± 2.15 FU/100 g), which is related to the higher fructosamine content found in these biscuits. The content of total AGEs presented a similar behaviour. The content of total AGEs was 4-fold higher in CSB (721.7 ± 50.33 U/100 g) indicating that the presence of sucrose in biscuits during baking increases the content, not only of initial MRPs, but also of the advanced ones. However, early MRPs often comprise the major proportion of the compounds formed (Poulsen et al., 2013). Comparisons among foods are difficult because of the multiplicity of methods and units with which AGEs are quantified. Nonetheless, it should be noted the feasibility of innovative biscuits and its ingredients when reducing the content of potentially harmful compounds. Sucrose replacement and SGC use entailed a decrease of all the MRPs measured. As explained, in absence of reducing sugars, Maillard reaction does not take place. Likewise, the content of CGA and other antioxidants present in coffee, and therefore in SGC, might also prevent this reaction (Fernandez-Gomez et al., 2015; Mesías et al., 2014). Hence, the innovative biscuits presented enhanced nutritional quality and safety.

3.2.2. Sensory analysis of biscuits

Colour, texture and taste were evaluated as presented in Table 5. All attributes were given a score higher than four. As regards colour, B2, B4 and B6 biscuits received the highest points, with similar scoring B6 and C2 commercial biscuit ($p > 0.05$). Results relative to texture and taste showed that commercial biscuits and B2 and B4 innovative biscuits did not present significant differences ($p > 0.05$).

Previous studies reported a decrease in the score for colour, texture and taste when stevia content increased. However, replacement of 20% sugar by stevia scored the highest values for these attributes (Kulthe, Pawar, Kotecha, Chavan, & Bansode, 2014) and 100% stevia in biscuits showed the maximum score for texture (Vatankhah, Garavand, Elhamirad, & Yaghbani, 2015). The use of poly-alcohols as sweeteners in biscuits was also evaluated by sensorial analysis (Zoulias, Piknis, & Oreopoulou, 2000) and maltitol presented the best score for flavour attribute. Our results are in line with these data.

For overall acceptance, as can be observed in Fig. 1, those innovative biscuits containing SCG, oligofructose and stevia or stevia and maltitiol, or maltitol (B2, B4 and B6, respectively) in

Table 5
Sensory evaluation (1–7 scale) of different biscuit formulations: B1 – 100% Stevia (ST) and spent coffee grounds (SCG); B2 – 100% ST, oligofructose (OF) and SCG; B3 – 30% ST, 70% Maltitol (MT) and SCG; B4 – 30% ST, 70% MT, OF and SCG; B5 – 100% MT and SCG; B6 – 100% MT, OF and SCG; C1 – MT and 4% fibre and C2 – MT, Isomaltitol and 8% fibre. B1, B3 and B5 are potentially "source of fibre" (⩾3 g fibre/100 g) and B2, B4 and B6 with "high fibre content" (⩾6 g fibre/100 g).

	Biscuit formulations							
	C1	C2	B1	B2	B3	B4	B5	B6
Colour	6.50 ± 0.64^{a}	$6.30 \pm 0.78^{a,e}$	$4.80 \pm 1.48^{b,d}$	5.50 ± 1.14^{c}	4.40 ± 1.06^{b}	$5.30 \pm 0.97^{c,d}$	4.50 ± 1.21^{b}	$5.70 \pm 1.31^{e,c}$
Texture	$6.40 \pm 0.66^{a,c}$	5.90 ± 1.02^{a}	4.20 ± 1.50^{b}	$5.10 \pm 1.26^{b,c,d}$	$4.80 \pm 1.39^{b,d}$	$5.70 \pm 1.05^{a,d}$	4.80 ± 1.48^{b}	$5.30 \pm 1.46,^{d}$
Taste	$5.80 \pm 1.00^{a,c}$	5.90 ± 1.24^{a}	4.70 ± 1.29^{b}	$5.20 \pm 1.17^{b,c}$	$4.90 \pm 1.23^{b,d}$	$5.40 \pm 0.94^{a,c,d}$	4.60 ± 1.27^{b}	$5.10 \pm 1.45^{b,d}$

Data are expressed as mean ± standard deviation (n = 26). In each row, values with different superscript letters are significantly different ($p < 0.05$).

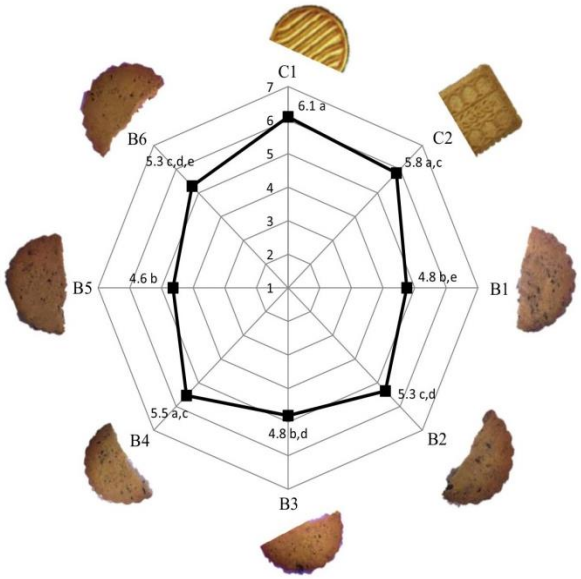

Fig. 1. Spider-web diagram which shows mean scores (n = 26) of 1–7 scale for overall acceptance of innovative sugar-free (B1–B6) and commercial (C1 and C2) biscuits and their external appearance. Different letters denote significant differences ($p < 0.05$). Formulations were as follows: B1 – 100% Stevia (ST) and spent coffee grounds (SCG); B2 – 100% ST, oligofructose (OF) and SCG; B3 – 30% ST, 70% Maltitol (MT) and SCG; B4 – 30% ST, 70% MT, OF and SCG; B5 – 100% MT and SCG; B6 – MT 100%, OF and SCG, C1 – MT and 4% fibre, and C2 – MT, Isomaltitol and 8% fibre-. B1, B3 and B5 are potentially "source of fibre" (⩾3 g fibre/100 g) and B2, B4 and B6 with "high fibre content" (⩾ 6 g fibre/100 g).

their formulation were scored significantly higher ($p < 0.05$) than their respective biscuits without oligofructose (B1, B3, B5).

Caffeine from coffee and steviol glycosides from stevia exert long-lasting bitter after-taste in mouth (Hellfritsch, Brockhoff, Stähler, Meyerhof, & Hofmann, 2012) producing occasionally undesirable effects in food matrix. Bitter aftertaste of stevioside is very persistent and appears in a dose dependent manner limiting its use at high concentrations.

Oligofructose presents slightly sweetness and therefore might act as enhancer of the taste in the innovative biscuits, masking negative off-flavours from stevia and SCG. Incorporation of oligofructose in biscuits also contributes to the balance of soluble:insoluble dietary fibre and promotes the growth of specific beneficial gut bacteria (prebiotic). Recently, relationship between non-digestible carbohydrates, including oligofructose, and reduction of post-prandial glycaemic responses was stablished by EFSA (2014). In this sense, oligofructose becomes a fundamental ingredient of these biscuits formulations.

Regarding, the overall acceptability of the innovative biscuits on the market (Fig. 1), a comparison with commercial biscuits was performed. Biscuits containing oligofructose (B2, B4 and B6) did not show significant differences ($p > 0.05$) with those commercial biscuits (C1 and C2) presenting high consumer adhesion and similar characteristics to the innovative biscuits, in terms of incorporated hypocaloric sweeteners and dietary fibre content. The commercial (C2) and the novel biscuits (B2, B4 and B6) might be included inside the "high fibre content" nutritional claim approved by the European Commission Regulation (EU) No 1924/2006, according to estimated dietary fibre values (Table 1).

Therefore, we could consider SCG and stevia and/or maltitol highly-accepted ingredients to include in innovative biscuits, and above all when combined with oligofructose in the formulation. Solid finished products were obtained according with consumers' preferences.

4. Conclusion

SCG from the industrial instant coffee are natural source of antioxidant insoluble dietary fibre, proteins, essential amino acids and low glycaemic sugars. SCG (4% w/w) can be used directly as food ingredient in solid foods such as biscuits without affecting

the conventional food preparation and the final quality of the product. These food formulations might be destined to people with reduced energetic intake and particular requirements. The application of SCG which we propose represents a value-added opportunity for coffee by-products utilization at a very low cost.

Acknowledgements

This study was funded by SUSCOFFEE (AGL2014-57239-R) project. The present research is part of the PhD. Thesis of Martinez-Saez, N. FPI-predoc fellow belongs to Autonomous University of Madrid (UAM), Spain. Rebollo-Hernanz, M. thanks the JAE Intro fellowship (JAEINT_15_00086) and the FPU predoctoral program of the Ministry of Education, Culture and Sports (FPU15/04238). Authors also thank Prosol for the spent coffee grounds and BENEO-ORAFTI for the FOS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.07.173.

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Study 3: *In vitro* assessment of the satiety effect of non-added sugar biscuit containing stevia, coffee fibre and fructooligosaccharides

Nuria Martinez-Saez [1], Christina M. Hochkogler [2], Veronika Somoza[2] and M. Dolores del Castillo [1]

[1]Institute of Food Science Research (CIAL, UAM-CSIC). Department of Bioactivity and Food Analysis. Food Bioscience Group. C/ Nicolás Cabrera, 9. Campus de Cantoblanco. Universidad Autónoma de Madrid. 28049. Madrid, Spain;
[2]Department of Nutritional and Physiological Chemistry, Christian Doppler Laboratory for Bioactive Aroma Compounds, Faculty of Chemistry, University of Vienna, Vienna, Austria;

Nutrients, *submitted on the 27th of April, 2017*

Nuria Martinez Saez

De:	sally.cheng@mdpi.com en nombre de Annie Zhang <annie.zhang@mdpi.com>
Enviado el:	viernes, 28 de abril de 2017 3:23
Para:	Maria Dolores Del Castillo
CC:	Annie Zhang; Nuria Martinez-Saez; Christina Maria Hochkogler; Veronika Somoza; Nutrients Editorial Office
Asunto:	[Nutrients] Manuscript ID: nutrients-196198 - Assistant Editor Assigned

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Journal: Nutrients
Manuscript ID: nutrients-196198
Title: In vitro assessment of the satiety effect of non-added sugar biscuit containing stevia, coffee fibre and fructooligosaccharides
Authors: Nuria Martinez-Saez , Christina Maria Hochkogler , Veronika Somoza , María Dolores Del Castillo *
Received: 27 April 2017
E-mails: nuria.m.s@cial.uam-csic.es, christina.maria.hochkogler@univie.ac.at, veronika.somoza@univie.ac.at, mdolores.delcastillo@csic.es

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Ms. Annie Zhang
Assistant Editor

-
Ms. Annie Zhang
MDPI Branch Office, Beijing
8th Floor, Aerospace Cooperation Building,
No.99 Zhongguancun East Road, Haidian District, Beijing, 100190, China
Email: annie.zhang@mdpi.com
Tel./Fax: +86 10 6280 0830

Article

In vitro assessment of the satiety effect of non-added sugar biscuit containing stevia, coffee fibre and fructooligosaccharides

Nuria Martinez-Saez [1], Christina M. Hochkogler [2], Veronika Somoza[2] and M. Dolores del Castillo [1],*

[1]Institute of Food Science Research (CIAL, UAM-CSIC). Department of Bioactivity and Food Analysis. Food Bioscience Group. C/ Nicolás Cabrera, 9. Campus de Cantoblanco. Universidad Autónoma de Madrid. 28049. Madrid, Spain; nuria.m.s@cial.uam-csic.es, mdolores.delcastillo@csic.es;

[2]Department of Nutritional and Physiological Chemistry, Christian Doppler Laboratory for Bioactive Aroma Compounds, Faculty of Chemistry, University of Vienna, Vienna, Austria; veronika.somoza@univie.ac.at

*Correspondence: mdolores.delcastillo@csic.es; Tel.: +34 91 0017900 Ext. 953

Abstract: This study assessed the *in vitro* effects of the bioaccessible food components released during the simulated human digestion of a coffee fibre-containing biscuit (CFB) on α-glucosidase activity, antioxidant capacity and satiety hormones. This biscuit presented a significantly ($p < 0.05$) lower glycaemic sugar content (60.6 mg/g) and a higher antioxidant capacity (15.1 mg chlorogenic acid/g) than a sucrose-containing biscuit (SCB). The CFB significantly reduced ($p < 0.05$) α-glucosidase activity (IC50 = 3.3 mg/ml) compared to the SCB (IC50 = 6.2 mg/ml). Serotonin and glucagon-like peptide-1 (GLP-1) release by differentiated Caco-2 and HuTu-80 cells, respectively, were stimulated by the CFB (354.7 ± 42.7% and 277.9 ± 14.1%) to the same order of magnitude as those of the SCB. In conclusion, the CFB was demonstrated to reduce simple sugar bioaccessibility and to improve satiety.

Keywords: α-glucosidase; biscuits; coffee fibre; fructoologosaccharides; GLP-1; serotonin; stevia; non-nutritive sweeteners.

1. INTRODUCTION

Consumption of energy dense foods high in fat and sugar is associated with the prevalence of obesity, type 2 diabetes, cardiovascular diseases and several cancers [1]. There is solid evidence that the risk of becoming overweight or obese is lower when the daily intake of free sugars is less than 10% of the total energy [2]. Increasing the price of unhealthy foods through sugar taxes could

potentially discourage the overconsumption of sugar [1]. However, multiple strategies are needed to battle obesity and associated comorbidities, e.g. diabetes.

Consumption of foods with a low glycaemic index (GI) can help regulate blood glucose levels, improve satiety, and control body weight [3]. Recently, high-fibre and sugar-free foods containing stevia as a non-nutritive sweetener have been developed by our group.[4] Stevia glycosides can help to optimize blood sugar and insulin levels in diabetics [5]. Foods high in fibre have been demonstrated to lower the GI as, for instance, galactomannan which slows gastric emptying and inhibits diabetes-related digestive enzymes [6,7].

The α-glucosidase is a membrane-bound intestinal enzyme, essential for degrading oligosaccharides to monosaccharides [8]. Food constituents such as dietary fibres, stevia and coffee phenols have been shown to act as glucosidase inhibitors [5,7,9], and to reduce postprandial hyperglycaemia. However, results on these compounds present in complex food matrices are missing.

Intake of satiety-inducing foods becomes a strategy to reduce sugar intake and related chronic diseases. Food ingestion activates the secretion of several gut-derived mediators [10], including the hormones localized in intestinal enterochromaffin cells, serotonin and glucagon-like peptide-1 (GLP-1). GLP-1 plays a role in the regulation of food intake and presents glucometabolic effects and serotonin is implicated in the control of satiety [10,11].

The goal of this study was to elucidate whether a replacement of sucrose by stevia, coffee fibre and fructooligosaccharides (FOS) in a sucrose-containing biscuit (SCB) improves its antioxidant capacity and outcome measures satiety *in vitro*. We report novel *in vitro* data on the potential use of a coffee fibre-containing biscuit (CFB) suitable for diabetics, people on a weight control diet and healthy people interested in maintaining a healthy body weight. This study also provides new evidence for using coffee fibre as a sustainable functional food ingredient.

2. MATERIALS AND METHODS

2.1. Chemicals

Bradford reagent was provided by Bio-Rad Laboratories S.A. The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA): α-amylase from human saliva (type IX-A), porcine pepsin from gastric mucosa (3.200-4.500 U/mg protein), pancreatin from porcine pancreas, porcine bile extract, bovine serum albumin (BSA), chlorogenic acid (CGA) (3-CGA), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS)), 6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic

acid (Trolox), phenol 5% (w/v), potassium persul¬phate, Folin-Ciocalteu reagent, Nα-Acetyl-L-lysine, ortho-phthalaldehyde (OPA), α-glucosidase from intestinal acetone powders of rat, 4-methylumbelliferyl α-D-glucopyranoside (4-MUG), acarbose, 3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyltetrazolium bromide (MTT) and trypan blue solution. We used the D-mannose/D-fructose/D-glucose assay kit and galactomannan assay kit from Megazyme International Ireland Ldt. (Ireland), Multi Species GLP-1 Total ELISA kit (Cat-nº. EZGLP1T-36K) from EMD Millipore (Missouri, USA) and Serotonin High Sensitive ELISA kit (Cat-nº. EA630/96) from DLD Diagnostika GmbH (Hamburg, Germany). For cell culture, Dulbecco's modified Eagle's medium (DMEM), minimum essential medium Eagle (MEM), L-glutamine, penicil¬lin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was from GIBCO Invitrogen (Karlsruhe, Germany). Water was purified using the Milli-Q and Elix system. All other chemicals and reagents were of analytical grade.

2.2. Apparatus and materials

BioTek powerWaveTM XS (BioTek Instruments, U.S.A), FP-6200 (JASCO, Easton, U.S.A) and Infinite® 200 PRO multimode (TECAN, Deutschland GmbH) microplates readers, convection oven (Romag S.A, Barcelona, Spain), UN 500 universal oven (Memmert, Germany) and Telstar Lyobeta-15 lyophilizer (Telstar, Spain) were used for analyses. For the cell assays, a Neubauer cell counting chamber (0.100 mm depth, 0.0025 mm2) (Paul Marienfeld GmbH & Co. KG, Germany), an incubator (BINDER GmbH, Germany), a Thermo Scientific™ MSC-Advantage™ class II biological safety cabinet (Thermo Fisher Scientific, USA), an autoclave (3870EA, Tuttnauer, USA), a TR400-SW TRINO microscope (VWR, Austria), CELLSTAR® multiwell culture plates and standard cell culture flasks (Greiner Bio-One GmbH, Austria) were used.

2.3. Food ingredients

Spent coffee grounds (SCG) from Robusta instant coffee were used as a source of antioxidant coffee fibre [4]. SCG were supplied by Prosol S.A (Spain) and stored at -20°C until use. FOS powder (ORAFTI®P95) was from Beneo-Orafti and stevia sweetener powder, containing 3% steviol glycosides, was purchased at a local supermarket. All other basic ingredients were purchased at specialized and certified food markets.

2.4. Food samples

Biscuit formulations can be seen in Table 1. Biscuits were prepared following Martinez-Saez et al. [4]. Briefly, the dough was prepared by mixing salt, baking powder and sucrose or stevia. Mineral

water was added to the dry mixture and thoroughly blended. In a separate bowl, lecithin and oil were mixed and then added to the mixture. Finally, the flour, coffee fibre and FOS were gradually added and the dough was kneaded. The dough was allowed to rest for 30 min, and shaped into discs. The biscuits were baked at 185º C for 16 min in an air recirculation oven. Two sets of 3 biscuits were baked in duplicate (n = 6). The biscuits were placed in the centre of the tray in order to reduce process variability during baking. Biscuits were grounded to obtain a representative sample for further analyses.

Table 1. Biscuit formulations: sucrose-containing biscuit (SCB) and coffee fibre-containing biscuit (CFB) made with stevia, coffee fibre and fructooligosaccharides (FOS).

INGREDIENTS (g)	SCB	CFB
Wheat flour	56.0	59.4
Water	20.0	21.2
Sunflower oil	7.75	8.20
Baking powder	0.55	0.58
Salt	0.37	0.39
Soy lecithin	0.33	0.35
Sucrose	15.0	-
Stevia	-	2.10
FOS	-	3.50
Coffee fibre	-	4.20
TOTAL dough	100	100
Estimated fibre content (g fibre/100g biscuit)	1.90	7.50 [1]

[1] "High fibre content" (≥ 6 g fibre/100g)

Biscuits were digested in triplicate under *in vitro* oral gastrointestinal human digestion conditions [4]. Briefly, all three stages, salivary (pH 6.9, 10 ml, 5 min, 3.9 U α-amylase/ml, aerobic), gastric (pH 2, 13 ml, 90 min, 71.2 U pepsin/ml, aerobic), and abiotic duodenal step (pH 7, 16 ml, 150 min, 9.2 mg pancreatin and 55.2 mg bile extract/ml, aerobic) were performed in the same flask. Digests were then centrifuged and the soluble fractions containing bioaccessible compounds to be absorbed and metabolized were treated with cholestyramine resin (10% w/v) to remove the bile acids and mimic the human bile salt reabsorption. Soluble fractions were then frozen at -20°C, lyophilized and stored at room temperature until further analysis.

Coffee fibre, stevia and FOS were also digested *in vitro* following the above-described procedure to gain insight into their contribution to bioactive compounds.

2.5. Cell culture: Caco-2 and HuTu-80

The human colon cancer cell line (Caco-2) was cultured in DMEM supplemented with FBS (10%), L-glutamine (2%) and penicillin/ streptomycin (1%) at 37°C and 5% CO2. Cells were grown in 12-well plates until reaching confluence, approximately after 3 days. Cell differentiation was obtained by subsequent culture for a further 21 days including medium changes every two to three days. The enterocyte-like differentiated Caco-2 cells were then used for further studies on serotonin hormone release.

The human duodenal cancer cell line (HuTu-80) was cultured in MEM supplemented with FBS (10%), L-glutamine (2%) and penicillin/ streptomycin (1%) at 37°C and 5% CO2. Cells were grown in 24-well plates until reaching confluence, approximately after 1 day. HuTu-80 cells were not differentiated with enterocyte-like properties, and 24 hours after seeding, they were ready to be used for analyses on GLP-1 hormone release.

After thawing, both cells lines were passaged 3-4 times to give cells time to recover their normal growth rate.

2.6. Bioaccessibility of food components

Digests of coffee fibre, stevia and biscuits (SCB and CFB) were characterized.

2.6.1. Total carbohydrates

Total carbohydrates were determined using the phenol–sulphuric method as described by Masuko et al. with slight modifications [12]. Samples (20 µl) were mixed with concentrated sulphuric acid (93–98%) (61 µl) and phenol solution (5%, w/v) (18 µl) in a multi-well plate. After incubation at 90ºC for 5 min in a water bath, the microplate was cooled to room temperature and absorbance was measured at 490 nm. The calibration curve was constructed using glucose (0.1–0.9 mg/ml) as standard. Reagent blank and sample blank were also prepared and analysed in each set of samples. All measurements were performed in triplicate and results were expressed as mg glucose equivalents (eq.)/g digest.

2.6.2. Galactomannan

The procedure for determining galactomannan content was performed using an enzymatic kit following the manufacturer's instructions. The method was adapted to a micromethod format. Analyses were carried out in triplicate and results were expressed as mg/g digest.

2.6.3. Free sugars

Glucose, fructose and mannose contents were determined using an enzymatic kit following the manufacturer's instructions. The method was adapted to micromethod. The analysis was performed in triplicate. Results were expressed as mg glucose, mg fructose and mg mannose/g digest.

2.6.4. Soluble proteins and peptides

The Bio-Rad Protein Assay, based on the Bradford method in micro-method format, was used to determine proteins and peptides. Reagents were prepared according to manufacturer's instructions. Briefly, a solution of Bradford reagent (1 : 4 reagent : milli-Q water) was prepared and filtered. Ten µl of sample and 200 µl of Bradford solution were placed in a multi-well plate. After 5 min of incubation at room temperature, absorbance was measured at 595 nm. Sample blank and reagent blank were also analysed. BSA was used as standard (0.05 – 1 mg/ml). All measurements were performed in triplicate. Results were expressed as mg BSA eq./g digest.

2.6.5. Free amino groups

The release of amino acids by proteolysis of proteins forming wheat flour and coffee fibre was measured by the OPA assay following Go et al. [13]. OPA reagent was freshly prepared by dissolving 10 mg OPA in 250 µl ethanol (95%, v/v), 9.8 ml PBS (10 mM, pH 7.4) and 20 µl β-mercaptoethanol. The reaction was carried out in a 96-well microtest plate by mixing 10 µl sample, 140µl PBS and 100 µl OPA reagent. Fluorescence was read at 360 ± 40 nm excitation and 460 ± 40 nm emission wavelengths for 15 min at 37 ºC. The calibration curve was constructed using standard solutions of Nα-acetyl-L-lysine (0.025 – 1 mM). All measurements were performed in triplicate, and data were expressed as mg Nα-acetyl-L-lysine eq./g digest.

2.6.6. Total phenolic content

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method as described by Contini et al. [14], adapted to a micromethod format. Ten µl of sample and 150 µl of Folin–Ciocalteu solution were incubated at room temperature for 3 min and 50 µl of sodium bicarbonate were added. Reaction was run for 120 min at 37ºC, and absorbance was read at 735 nm. Sample blank and

reagent blank were also analysed in each set of samples. The CGA calibration curve (0.1-1 mg/ml) was used for quantification. Measurements were performed in triplicate and results were expressed as mg CGA eq./g digest.

2.6.7. Antioxidant capacity

The overall antioxidant capacity of the digested biscuits and coffee fibre was analysed using the indirect ABTS•+ decolourisation assay as described by Oki et al. [15]. An ABTS•+ stock solution was prepared by adding 140 mmol/l potassium persulfate (44 µl) to a 7 mmol/l ABTS•+ aqueous solution (2.5 ml), and the mixture was then allowed to stand for 16 h at room temperature. The working solution of the radical ABTS•+ was prepared by diluting the stock solution 1:75 (v/v) in sodium phosphate buffer (5 mmol/l, pH 7.4) to obtain an absorbance value of 0.7 ± 0.02 at 734 nm. Samples (30 µl) were added to ABTS•+ solution (270 µl) in a microplate. Absorbance was measured at 734 nm for 10 min at 30ºC. CGA (0.025-0.25 mmol/l) and trolox (0.025-0.25 mmol/l) were used for quantification. All measurements were performed in triplicate and results were expressed as mg CGA eq./g digest.

2.7. Health-promoting properties of bioaccessible food components

2.7.1. Alpha-glucosidase inhibition assay

The α-glucosidase inhibitory activity of the digested biscuits, coffee fibre, stevia and FOS was analysed following the methodology described by Berthelot et al. and Geddes et al. [16,17] with slight modifications. Alpha-glucosidase enzyme was extracted previous to the assay. Briefly, 100 mg of rat intestine powder were dissolved in 3 ml NaCl (0.9%), sonicated in an ice bath for 6 min and then centrifuged at 10000 g for 30 min. The supernatant containing the enzyme was stored in the freezer. In a 96-well microplate, 100 µl of sample dissolved in PBS (100 mM, pH 6.9) were mixed with 100 µl α-glucosidase (diluted 1/10) and 100 µl 4-MUG (2 mM). Fluorescence was then monitored at an excitation wavelength of 360 nm and an emission wavelength of 460 nm for 30 min at 37 °C. Blank of sample and negative control (buffer, enzyme and 4-MUG) were included. Acarbose was used as a positive control (standard inhibitor). Curves of samples and acarbose were assayed to cover the whole range of inhibition of the enzyme (≈0.5-96%). The percentage (%) of α-glucosidase inhibition was calculated using the equation:

$$\text{α-glucosidase inhibition (\%)} = \frac{F_{nc}-F_s}{F_{nc}} \times 100$$

Where Fn.c. is the fluorescence of the negative control (without inhibitor) and Fs is the fluorescence of the sample. All measurements were performed in triplicate. Results were expressed as the concentration causing 50% inhibition (IC50 mg/ml).

2.7.2. Assays of serotonin and GLP-1 secretion

Soluble fractions recovered from the digested SCB, CFB and coffee fibre containing bioaccessible compounds for absorption and metabolism were used to stimulate the secretion of satiating hormones in cell cultures. The effect of stevia on the release of satiating hormones was also tested.

- Cell viability: Cytotoxic effects of test samples were excluded by performing the colorimetric MTT assay [18]. Differentiated Caco-2 cells were incubated with samples (0.05, 0.5 and 5 mg/ml) diluted in PBS containing ascorbic acid (0.1%) and after a 5-min exposure to cells, the samples were removed. HuTu-80 cells were first starved with medium free of serum, glucose and glutamine for 1 h prior to the incubation with the samples (0.01, 0.05 and 0.5 mg/ml) diluted in the starving-medium. After 90-min exposure, samples were finally removed.

  In both cellular lines, exposure was carried out at 37ºC and a negative (medium) and positive control (DMSO) were also included. The MTT solution (50 mg/ml, 1:6) was left to incubate (10-15 min) and the resulting formazan diluted in DMSO was measured at 570 nm. Viability was determined relative to untreated negative control cells (100%). Three groups of different passaging and samples in duplicate were performed in each set of analysis (n = 3, tr = 6). The percentage of cell vitality was calculated as follows: vitality (%) = (dead cell number / total cell number) × 100. Results were expressed as total cell viability / control (T/C) [%].

- Stimulation and quantification of serotonin: Caco-2 cells were supplemented with the bioaccessible fractions (150 µl) at three different concentrations (0.5, 0.05, 0.01 mg/ml) in duplicate. Cells were washed with PBS prior to the addition of samples. The cells were then stimulated for 5 min in darkness in an orbital shaker. Supernatants were removed from the cells and frozen until further quantification of the serotonin hormone. Positive (cinnamaldehyde, 5 mM) and negative (buffer) controls were also tested.

  The serotonin released by Caco-2 cells was quantified in darkness using a highly sensitive enzyme immunoassay kit (ELISA competitive) following the manufacturer's instructions. Three groups of different passaging were performed in each set of analysis (n = 3, tr = 6). Results were expressed as T/C [%] compared to the control.

- Stimulation and quantification of GLP-1: HuTu-80 cells were first starved with medium free of serum, glucose and glutamine for 1 h prior to stimulation with the bioaccessible fractions (500 µl) at 0.01, 0.05 and 0.5 mg/ml in duplicate. Positive (glutamine 40 mM) and negative (medium) controls were also tested. Exposure to the cells was for 1 h 30 min at 37 ºC. After the stimulation period, supernatants were collected and frozen until further quantification of the GLP-1 hormone.

  The GLP-1 hormone released from HuTu-80 cells was quantified using the sandwich ELISA kit following the manufacturer's instructions. Three groups of different passaging were performed in each set of analysis (n = 3, tr = 6). Results were expressed T/C [%] compared to the control.

2.8. Statistical Analysis

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software Inc., California, USA). Data were expressed as the mean value ± standard deviation for all analyses except for those comprising culture cells which were expressed as mean ± standard error of the mean (SEM). Differences between means were determined through analysis of variance (ANOVA), one-way ANOVA followed by Dunn's, Fisher LSD or Holm-Sidak post-hoc tests. Differences were considered to be significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Bioaccessibility of food components

Table 2 shows data on the bioaccessible food components released during the digestion process. In the case of coffee fibre, the major component of the bioaccessible fraction of the digest were carbohydrates (11.3%), whereas only trace amounts of sugars such as glucose (0.01%), fructose (0.02%) and mannose (0.03%) were detected. In contrast, a higher content in polysaccharides like galactomannan (2%) was quantitated. TPC represented 1.56% of the bioaccessible food components, and proteins (0.88%) and free amino groups (2.2%) were also present. These results suggest that SCG used as coffee fibre mainly provides complex carbohydrates to the bioaccessible fraction, which is in accordance with the literature [4,19].

In the case of the SCB, the major components of the bioaccessible fraction of the digest were also carbohydrates (64.8%). Sugars represented 18% of total carbohydrates. Fructose (52.3%) and glucose (47%) were found in the highest amounts, followed by trace amounts of mannose (0.7%). Protein and amino acid contents were lower than 1% (0.41% and 0.80%, respectively) and TPC represented 0.85%. Wheat flour starch seems to be the main contributor to the carbohydrate content

of the biscuit (Table 1). According to the literature, wheat flour is composed of 65% digestible starch [20], which is hydrolysed by digestive enzymes -salivary and pancreatic α-amylases- to glucose molecules and oligosaccharides [21]. The sucrose added to the traditional formulation can also be converted into glucose and fructose, mainly by chemical reactions during the processing of cereal-based products [22], and the acid conditions of the stomach [23], since the intestinal sucrase enzyme was not used in this particular digestion model. These available carbohydrates may turn the traditional biscuit into a high GI food which may consequently cause fast postprandial blood glucose glycaemic responses [24].

High GI diets are associated with decreased satiety and increased glucose intolerance, a greater risk of overweight and obesity and impaired lipid metabolism [25]. Furthermore, glucose and fructose follow different metabolic pathways after their absorption, resulting in different effects on blood glucose concentrations. Obesity is related to an elevated intake of both sugars. Type 2 diabetes is associated with high glucose diets, while the overconsumption of fructose is associated with non-alcoholic fatty liver diseases and augmented de-novo triglyceride synthesis [26].

The formulation of the CFB by replacing sucrose with stevia, coffee fibre and FOS clearly favoured the contents of ingredients with beneficial effects on GI (Table 2). The bioavailability of glycaemic sugars was significantly ($p < 0.05$) lower with reductions of 46.4 ± 8.8% and 35.6 ± 1.1% for glucose and fructose, respectively, compared to the SCB. The stevia sweetener did not significantly contribute to the sugar content of the CFB (Table 2), as it was hydrolysed in a small amount (4%). Stevia, in particular steviol glycosides, cannot be hydrolysed by the digestive enzymes in the small intestine, but is metabolized to steviol by the microbiota of the colon [27]. However, the stevia added to the CFB contained digestible carbohydrate –maltodextrin (97%)- as an additive. Stevia plays an important role in lowering the GI of foods. The moderate glucose levels found in CFB are a necessary primary energy source for proper cell function in the organism [28], and have a direct positive impact on the GI of the biscuit. On the other hand, galactomannan was present in the CFB unlike the SCB. The coffee fibre included in the CFB is a natural source of galactomannan (Table 2). Intake of galactomannan is associated with reduced weight gain, adiposity, liver fat and blood glucose levels [29], making coffee fibre an attractive ingredient for confectioneries. Moreover, the FOS incorporated in the CFB as soluble fibre has been shown to reduce post-prandial glycaemic responses [30]. Marangoni & Poli [25], also obtained a markedly lower glycaemic index in bread and biscuits by adding a proprietary fibre mixture to their formulations.

Regarding the antioxidant properties of the biscuits, significant differences ($p < 0.05$) were found between the CFB and the SCB. In the bioaccessibility of antioxidants, estimated as the overall

antioxidant capacity of the food digests, the digestion of the CFB released a significantly greater amount of antioxidants (15.07 ± 1.45 mg CGA eq./g digest) than in the SCB (10.43 ± 0.90 mg CGA eq./g digest). Most of the antioxidants of the SCB may be ascribed to phenolic compounds (Table 2), in contrast, the CFB might also contain other non-phenolic antioxidants which may contribute to its overall antioxidant capacity. The bioaccessible fraction of the digested coffee fibre had a high antioxidant character (46.14 ± 3.61 mg CGA eq./g digest). Furthermore, stevia [31], FOS [32], and the gluten peptides released during the digestion process by chemical and enzymatic hydrolysis [33], may also exert an antioxidant character. The high antioxidant properties of the CFB may play an important role in reducing the risk of obesity and diabetes. Consequently, introducing food antioxidants through the diet may be of great interest. High-antioxidant diets have been related to reduced inflammation and increased circulating antioxidants in cross-sectional and randomized intervention studies [34].

Table 2. Bioaccessible compounds released during *in vitro* oral-gastrointestinal digestion of a sucrose-containing biscuit (SCB), a coffee fibre-containing biscuit (CFB), coffee fibre and stevia.

Bioaccessible compounds	SCB	CFB	Coffee fibre	Stevia
Total Carbohydrates				
mg glucose eq./g digest	647 ± 70.0^{a}	609 ± 17.2^{a}	113 ± 7.57^{b}	n.d.
Galactomannan				
mg/g digest	ND	1.60 ± 0.11^{a}	19.49 ± 1.19^{b}	n.d.
Sugars				
mg glucose/g digest	54.8 ± 9.94^{a}	28.8 ± 0.65^{b}	0.01 ± 0.0^{c}	40.0 ± 0.57^{d}
mg fructose/g digest	60.9 ± 3.31^{a}	39.2 ± 1.48^{b}	0.21 ± 0.01^{c}	6.20 ± 0.46^{d}
mg mannose/g digest	0.74 ± 0.11^{a}	0.59 ± 0.11^{a}	0.29 ± 0.01^{b}	ND
Soluble proteins				
mg BSA eq./g digest	4.11 ± 0.09^{a}	3.88 ± 0.30^{a}	8.75 ± 0.28^{b}	n.d.
Free amino groups				
mg Nα-acetyl-Lys eq. /g digest	7.95 ± 0.19^{a}	9.16 ± 0.83^{a}	24.0 ± 2.53^{b}	n.d.
Total phenolic content				
mg CGA eq./g digest	8.86 ± 0.49^{a}	8.98 ± 0.52^{a}	15.56 ± 0.95^{b}	n.d.

ND Not Detected. n.d. not determined. Data are presented as mean ± standard deviation. Triplicate of sample preparation and triplicate of analysis (n = 9). Different letters indicate significant differences ($p < 0.05$) between the samples of the same row.

3.2. Health-promoting properties of foods

3.2.1. Anti-diabetic properties

IC50 values for the α-glucosidase inhibition were calculated from dose–response curves (Figure 1 (a), (b) and (c)). The IC50 for acarbose was 4.4 µg/ml. Alpha-glucosidase inhibitors were detected in the digests of the samples. However, the content of α-glucosidase inhibitors released during digestion differed significantly ($p < 0.05$) between samples. The CFB presented the lowest IC50 value of all the studied samples (Table 3). According to the literature, the CFB is a better inhibitor than other food products such as lemon (IC50 36.59 mg/ml), lime (10.96 mg/ml), grapefruit (62.10 mg/ml) [35], green tea (11.1 mg/ml), sardine muscle hydrolizate (48.7 mg/ml) and yogurt (519.8 mg/ml) [36].

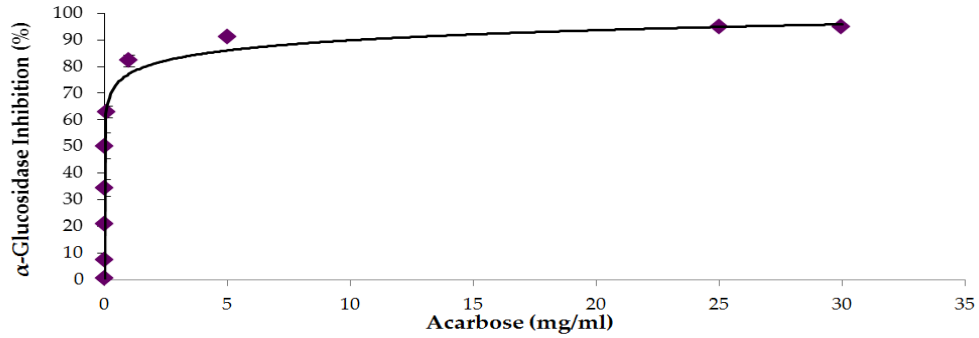
Stevia, FOS and coffee fibre are demonstrated to contribute to the inhibitory effect of the α-glucosidase observed for the CFB, although stevia had a higher inhibitory capacity than the other two ingredients. The inhibitory effect of stevia against α-glucosidase has been previously described [9]. Phenolic compounds released during the digestion of coffee fibre may also act as α-glucosidase inhibitors, as for instance the main phenolic compound of coffee, CGA with potential benefits on type 2 diabetes [37]. Other compounds released during the digestion of the CFB such as bioactive peptides could also exhibit anti-diabetic properties [38].

These results suggest the potential use of the CFB as a diabetic-friendly biscuit with great interest for people with type 2 diabetes and/or overweight or obese, as it has the potential to delay intestinal glucose absorption and enhance postprandial hyperglycaemia levels. However, proof-of-principle human intervention studies are needed to verify this effect.

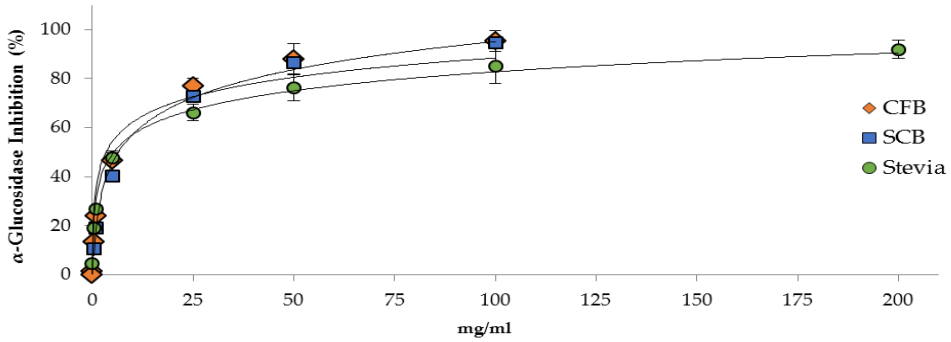
Table 3. IC50 values against α-glucosidase activity and equivalents of acarbose of the bioaccessible fractions of digested sucrose-containing biscuit (SCB), coffee fibre-containing biscuit (CFB), coffee fibre, stevia and fructooligosaccharides (FOS).

	IC50 (mg/ml)	mg acarbose eq./g digest
Acarbose	0.004 ± 0.00^{a}	-
Stevia	$5.53 \pm 0.35^{b,c}$	0.79 ± 0.05
SCB	6.22 ± 0.33^{b}	0.70 ± 0.04
CFB	3.32 ± 0.12^{c}	1.32 ± 0.05
Coffee fibre	23.9 ± 1.31^{d}	0.18 ± 0.01
FOS	53.4 ± 2.22^{e}	0.08 ± 0.00

Values represent mean ± standard deviation (n = 2). Differences were tested with one-way ANOVA followed by Fisher post-hoc test ($p < 0.05$) and marked with letters a, b, c, d and e



(**a**)



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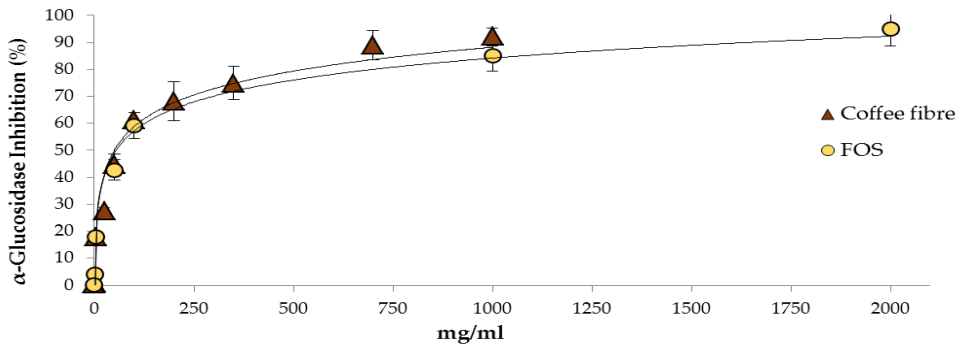

(**c**)

Figure 1. Effect on α-glucosidase activity represented by dose-response curves of (a) acarbose (0.1 µg/ml – 30 mg/ml), (b) soluble fractions recovered from the digested sucrose-containing biscuit (SCB) (0.5-100 mg/ml), coffee fibre-containing biscuit (CFB) (0.01-100 mg/ml) and stevia (0.05-200 mg/ml), and (c) soluble fractions recovered from the digested coffee fibre (0.5-1000 mg/ml) and FOS (0.5-2000 mg/ml). Values represent mean ± standard deviation. Duplicate of sample preparation and triplicate of analysis (n = 6).

3.2.2. Release of satiety hormones

Different doses of the tested samples were not cytotoxic (Figure S1, supplementary material). The effect of the samples on cellular secretion of serotonin is shown in Figure 2. Caco-2 cells treated with 0.5 mg/ml of the CFB digest exhibited a 4-fold increase in serotonin secretion (355 ± 42.7%). No differences in serotonin release were found ($p > 0.05$) between the digests of the SCB and the CFB. However, the compounds released during the digestion of the antioxidant coffee fibre had a significant effect on the secretion of serotonin, increasing serotonin release 7-fold (763 ± 81.4%) compared to the basal level. To the best of our knowledge, this is the first time that the effect of the antioxidant coffee fibre on satiety hormones has been reported. Gostner et al. found that coffee compounds such as gallic acid and cafeic acid had the potential to increase tryptophan availability, needed for the biosynthesis of serotonin, via inhibition of indoleamine 2,3-dioxygenase which is involved in tryptophan metabolism [39]. Further studies are required to elucidate which of the compounds released during the digestion of the coffee fibre is responsible for its satiating effect.

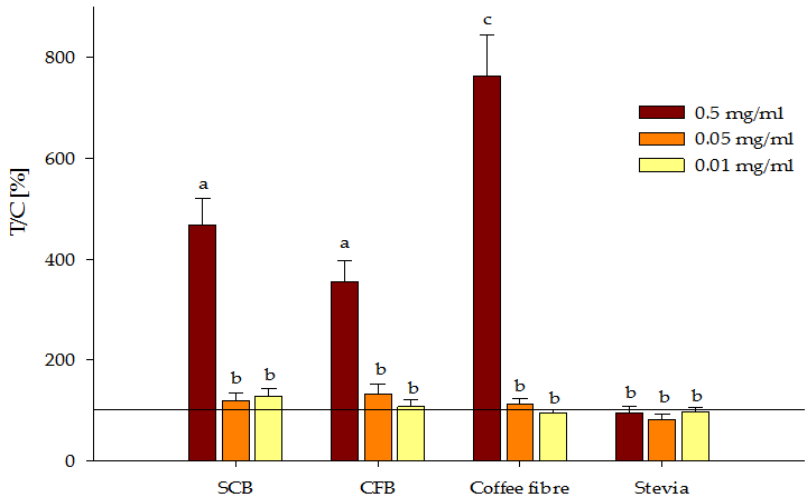

Figure 2. Serotonin release after stimulation of Caco-2 cells at 0.5, 0.05 and 0.01 mg/ml with soluble fractions recovered from the digested sucrose-containing biscuit (SCB), coffee fibre-containing biscuit (CFB) and antioxidant coffee fibre that contain bioaccessible compounds; as well as stevia. Results are displayed as T/C in percent compared to the control (cells with media = 100%). All measurements were expressed as mean ± SEM (n = 3, tr = 6). Significant differences between treatments were tested with one-way ANOVA followed by the Holm–Sidak posthoc test ($p < 0.05$) and marked with the letters a, b, c and d.

In contrast, the natural hypocaloric sweetener, stevia, did not stimulate the serotonin secretion in differentiated Caco-2 cells at the tested concentrations. This result is in concordance with that described by Ripken et al. did not found serotonin release from porcine intestinal tissue segments

when incubated with Rebaudioside A, the sweetest glycoside of the stevia [40]. Moreover, Anton et al. reported in human intervention that the intake of preloads containing stevia had satiety levels similar to those of sucrose preloads, measured by the subjective visual analogue scale [41]. Therefore, more investigations are required to clarify the role of this hypocaloric sweetener and its metabolites in serotonin secretion.

Other bioaccessible compounds may exhibit serotonin stimulation. For instance, gluten peptides from wheat flour have been shown to contribute to higher hypothalamic and cortical serotonin levels in animal models [42]. Phenolic compounds can also play an important role in appetite suppression by stimulating this important signalling molecule [43]. Furthermore, Maillard reaction products such as N(ε)-carboxymethyl lysine from heat-treated food like biscuits, have been found to contribute to satiety regulation through central/brain serotonin release in SH-SY5Y cells [44]. Therefore, biscuits made using sucrose as a sweetener might contain these advanced products of the Maillard reaction that could contribute to the stimulation of serotonin. However, replacing sucrose with stevia limits the progress of Maillard reaction in food toward advanced stages [45], and may not significantly contribute to a postprandial satiating effect of the food prepared according to the CFB. Despite the controversial effect of peripheral serotonin on satiety [46], there is evidence that peripheral serotonin unfolds similar effects on satiety than central serotonin [11,47]. Further studies are needed to identify the individual food components that contribute to the release of serotonin from the gut in the CFB and the SCB.

Regarding cellular secretion of GLP-1, significant GLP-1 values were obtained ($p < 0.05$) for the stimulation of HuTu-80 cells with the bioaccessible compounds released during the digestion of food formulations (Figure 3). Compared to the non-treated control cells, GLP-1 release was significantly higher ($p < 0.05$), i.e. three times higher (278 ± 14.1%), after incubation of HuTu-80 cells with the soluble fraction of the digested novel CFB (0.05 mg/ml). No significant differences were found ($p > 0.05$) between the SCB and the CFB. The same trend was observed for all concentrations of the biscuits. The compounds released during the digestion of antioxidant coffee fibre from SCG significantly stimulated the secretion of the GLP-1 hormone. Although researchers have previously reported GLP-1 stimulation caused by coffee beverages in cells, mice and humans,[48] this is the first report of the effect of coffee fibre from SCG.

On the other hand, stevia was not effective at the three tested concentrations, which is in agreement with the results of Fujita et al. presented for an *in vivo* animal model [49]. FOS present in the CFB (Table 1) has been associated with appetite suppression via stimulation of GLP-1 release [50]. Other bioaccessible compounds, such as proteins and peptides, may also stimulate GLP-1 release. For instance, intact and digested wheat proteins have been found to produce satiety and have an anti-diabetic effect through GLP-1 stimulation [51]. Further studies are required to characterize the food compounds released during digestion and to determine those which contribute to the satiating effect of the biscuits.

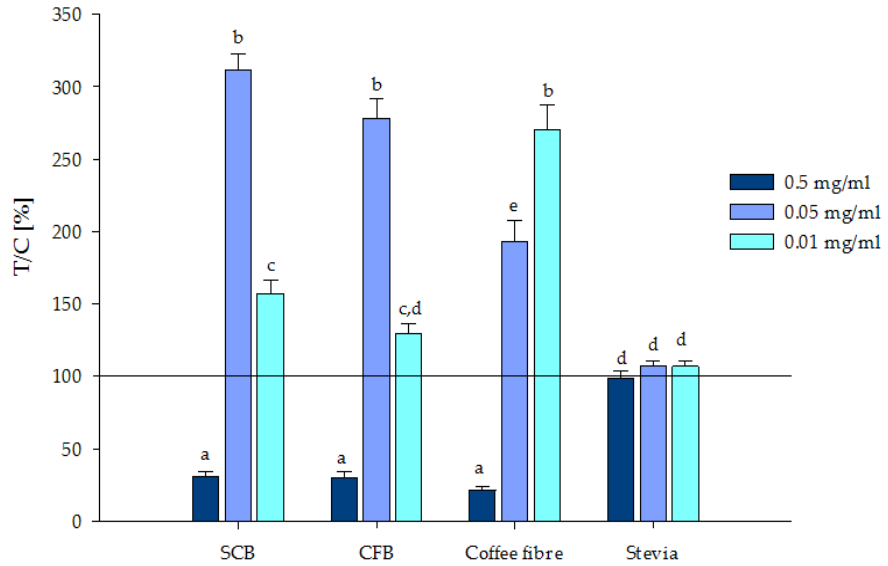

Figure 3. Glucagon-like peptide-1 (GLP-1) release after stimulation of HuTu-80 cells at 0.5, 0.05 and 0.01 mg/ml soluble fractions recovered from the digested sucrose containing biscuit (SCB), coffee fibre containing biscuit (CFB) and antioxidant coffee fibre that contain bioaccessible compounds; as well as stevia. Results are displayed as T/C in percent compared to control (cells with media = 100%). All measurements were expressed as mean ± SEM (n = 3, tr = 6). Significant differences between treatments were tested with one-way ANOVA followed by the Holm–Sidak posthoc test ($p < 0.05$) and marked with the letters a, b, c and d.

4. CONCLUSIONS

To the best of our knowledge, this is the first report of anti-diabetic and satiating effects of bioaccessible compounds released during the digestion of a CFB and their ingredients, antioxidant coffee fibre, FOS and stevia. New evidence of the use of antioxidant coffee fibre as a sustainable functional food ingredient has been provided.

Acknowledgments: This study was funded by the SUSCOFFEE (AGL2014-57239-R) project and carried out in collaboration with the University of Vienna. Martinez-Saez, N. thanks the Autonomous University of Madrid (UAM), Spain, for her FPI-predoc fellowship and the financial support for her short stay at the University of Vienna. The authors also thank Prosol for supplying spent coffee grounds and BENEO-ORAFTI for donating FOS. Moreover, the financial support by the Austrian Federal Ministry of Economy, Family and Youth and the Austrian National Foundation for Research, Technology and Development is gratefully acknowledged.

Author Contributions: "del Castillo, M.D. and Somoza, V. designed the experiments, supervised the investigation and revised the manuscript. Martinez-Saez, N. and Hochkogler, C.M. performed the experiments and analysed the data; Martinez-Saez, N. is the principal author of the investigation since it is part of her PhD thesis, supervised by del Castillo, PhD."

Conflicts of Interest: Declare conflicts of interest or state "The authors declare no conflict of interest.

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Supplementary material

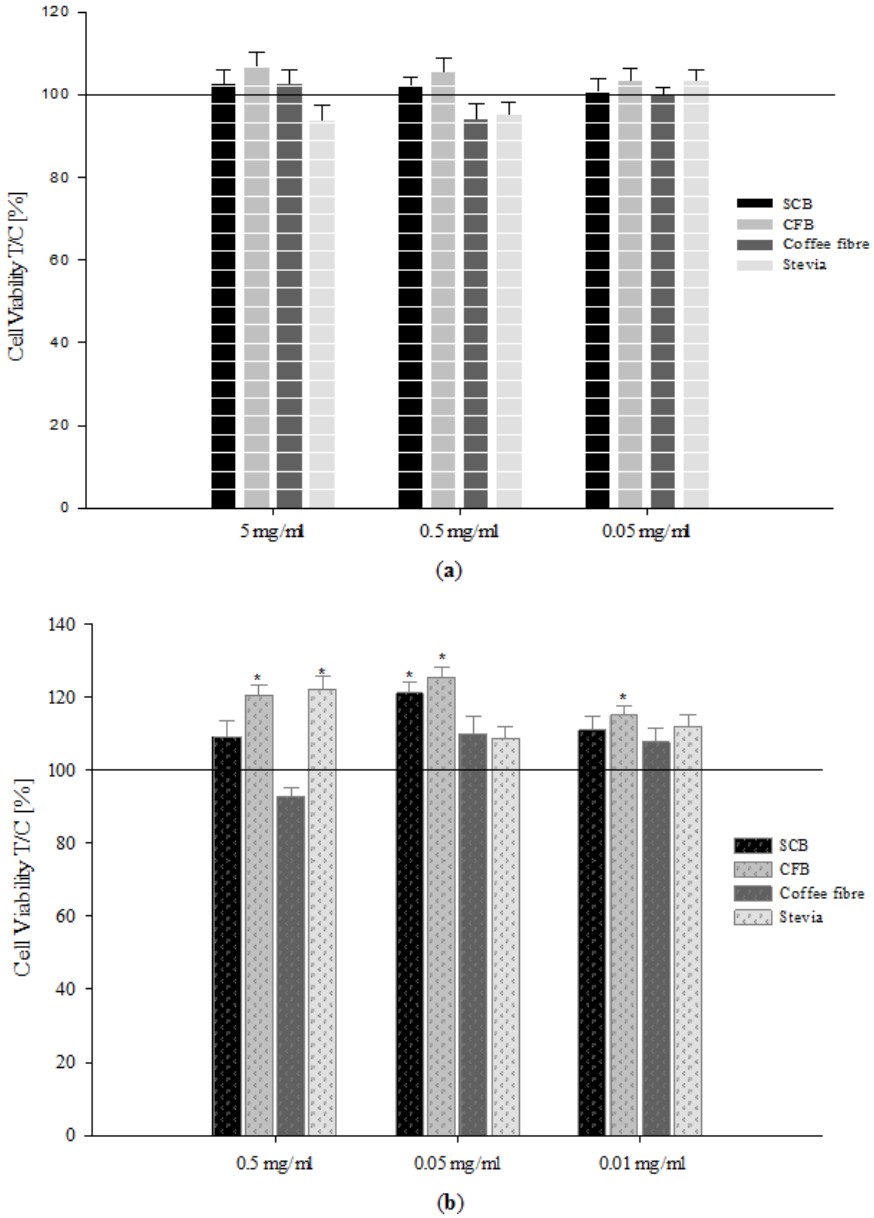

Figure S1. Cytotoxic effects of soluble fractions recovered from the digested sucrose-containing biscuit (SCB), coffee fibre-containing biscuit (CFB) and antioxidant coffee fibre that contain bioaccessible compounds; as well as, stevia, on (a) Caco-2 cells at concentrations of 5, 0.5 and 0.05 mg/ml, and on (b) HuTu-80 cells at 0.5, 0.05 and 0.01 mg/ml, compared to control (cells with media, 100% viability). All measurements were expressed as mean ± SEM (n=3, tr=6). Significant differences vs. control were determined by One-Way ANOVA followed by Dunn's posthoc test ($p < 0.05$) and marked as '*'.

CHAPTER 3

Impact of digestive process on the formation of potential harmful compounds

This chapter presents evidence on the formation of compounds with health impact during the digestive process. *In vitro* abiotic gastrointestinal digestion of simplified meal systems was performed and the formation of MRPs associated to the pathogenesis of diabetes and its complications was examined.

- **Study 1**: Martinez-Saez, N.; Fernandez-Gomez, B.; Weijing, C.; Uribarri, J. & del Castillo, M.D. ***In vitro* formation of Maillard reaction products (MRPs) during simulated digestion of food models corresponding to average and sugar-containing meals.** This article has been submitted to Food Research International on the 21st of My, 2017.

Study 1: *In vitro* formation of Maillard reaction products (MRPs) during simulated digestion of food models corresponding to average and sugar-containing meals

Nuria Martinez-Saez[1], Beatriz Fernandez-Gomez[1], Weijing Cai[2], Jaime Uribarri[2], María Dolores del Castillo[1].

[1] Food Bioscience Group, Department of Bioactivity and Food Analysis, Institute of Food Science Research (CIAL, UAM-CSIC). C/ Nicolás Cabrera, 9, Campus de Cantoblanco, Universidad Autónoma de Madrid, 28049, Madrid, Spain.
[2] Department of Medicine, The Icahn School of Medicine at Mount Sinai, 1468 Madison Ave, New York, NY 10029, USA.

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TITLE: ***In vitro* formation of Maillard reaction products (MRPs) during simulated digestion of food models corresponding to average and sugar-containing meals**

AUTHORS: Nuria Martinez-Saez[1], Beatriz Fernandez-Gomez[1], Weijing Cai[2], Jaime Uribarri[2], María Dolores del Castillo[1].

AFFILATIONS:

[1] Food Bioscience Group, Department of Bioactivity and Food Analysis, Institute of Food Science Research (CIAL, UAM-CSIC). C/ Nicolás Cabrera, 9, Campus de Cantoblanco, Universidad Autónoma de Madrid, 28049, Madrid, Spain. www.cial.uam-csic.es. E-mail: nuria.m.s@cial.uam-csic.es, mdolores.delcastillo@csic.es

[2] Department of Medicine, The Icahn School of Medicine at Mount Sinai, 1468 Madison Ave, New York, NY 10029, USA.

Corresponding author: Dr. M. Dolores del Castillo[1], Tel: +34 91 0017900 Ext. 953, Fax: +34 91 0017905, e-mail: mdolores.delcastillo@csic.es

Keywords: MRPs, AGEs, digestion, average meal, sugar-containing meals, malabsorption, delayed sugar absorption, amino acids bioavailability

Abbreviations: AGEs, Advanced glycation end products; **BSA,** Bovine serum albumin; **CML**, Carboxymethyl-lysine; **DMF**, 1-deoxy-1-morpholinofructose; **ELISA**, enzyme-linked immunosorbent assay; **HFCS**, High fructose corn syrup; **MGO**, Methyl glyoxal; **MR**, Maillard reaction; **MRPs**, Maillard reaction products; **NBT**, nitroblue tetrazolium; **RFU**, relative fluorescence units.

Abstract

The aim of the present research was to study the formation of Maillard reaction products (MRPs) during digestive process of simplified meal systems. An average meal (protein, starch and oil) and sugar-containing meals (protein and glucose or fructose or high fructose corn syrup (HCFS)) were tested. Intestinal simple amino acid systems were also analyzed to gain insight into their contribution to the Maillard reaction (MR). Decrease of lysine (11.7-34%), arginine (24-35%) and other amino acids occurred after digestion of the meals. Fructosamine (42.6±4.7 and 332.9±10.4 μg/ml) and fluorescent adducts (22270±119.6 and 9283±188.3 RFU) were detected in digests of those meals containing HCFS and starch, respectively. Carboxymethyllysine (CML) (5.03±1.09 μg/ml) and MGO-derivative AGEs (12.2±1.5 μg/ml) were found in the meals composed of fructose and only MGO-

derivative AGEs (12.2±1.6 µg/ml) in presence of glucose. Physiological concentrations (43 mM) of sugars in simplified systems composed by single amino acids caused formation of MRPs under intestinal conditions. Arginine and fructose (314 mM) showed formation of fructosamine and different AGEs. Fructose (43 mM) gave rise to CML by interaction with lysine, which was observed within 1 hour of incubation at intestinal conditions. This may be feasible under the conditions of fructose malabsorption. Results suggested the interest of the use of meal systems for a better understanding of complex chemical events taking place during digestion such as MR. This is the first study proposing the formation of non-fluorescent AGEs associated to the pathogenesis of diabetes during digestion of sugar containing and average meals. Their formation may be possible under those conditions where sugar absorption is delayed such as fructose malabsorption or the intake of a fatty meal. The occurrence of the MR during the digestion process may reduce the bioavailability of essential amino acids and increase the production of MRPs causing health disorders.

1. INTRODUCTION

The Maillard reaction (MR) is a series of non-enzymatic reactions between reducing sugars and proteins firstly described in foods during thermal processing and long-term storage. MR products (MRPs) provide sensory attributes of cooked foods, and are the main determinants of the consumer's quality-oriented food choice. Early products of the MR (Amadori and Heyns compounds) tend to decline after overheating and give rise to advanced glycation end products (AGEs) (Mesías & Delgado-Andrade, 2017).

The modern diet is a large source of MRPs (Goldberg et al., 2004; Uribarri et al., 2010; Vlassara & Uribarri, 2004) significantly contributing to those endogenously produced. Previous studies have showed that dietary AGEs are partially absorbed (Cai et al., 2008; Lin et al., 2003), approximately 10–30% (Uribarri et al., 2007), and they are indistinguishable from endogenous AGEs (Cai et al., 2002). Great interest has been raised due to their association with oxidative stress and inflammation, processes that eventually cause most chronic diseases, including diabetes, and cardiovascular disease (Sayej et al., 2016; Vlassara & Uribarri, 2014). Recently, a relationship has been reported between the intake of high fructose corn syrup (HFCS) sweetened soft drinks, fruit drinks and apple juice and the development of arthritis (DeChristopher, Uribarri, & Tucker, 2016), asthma (DeChristopher, Uribarri, & Tucker, 2015b) and chronic bronchitis (DeChristopher, Uribarri, & Tucker, 2015a). The authors hypothesized that these associations might be mediated through the intestinal in situ formation of fructose-AGEs and their subsequent absorption. This event may be favored by the increased intake of beverages and food containing HFCS (Ventura, Davis, & Goran, 2011) in which the ratio of fructose to glucose is higher than 1:1 therefore creating conditions for potential

fructose malabsorption and intraluminal generation of fructose-AGEs (DeChristopher et al., 2016). In fact, malabsorption of simple carbohydrates affects 20% to 30% of the European population (Raithel et al., 2013). The dietary lipid content can affect the intestinal membrane function (Thomson, 1982) thus attenuating intestinal sugar uptake and therefore potentially creating transient increase in intraluminal concentration of sugars Moreover, the natural conditions of the digestive tract (pH, minerals, temperature) might be a favorable environment for the MR to take place (Nursten, 2005). Based on the above information, the need to study the effect of gastrointestinal digestion process on the MRP formation, a largely unexplored area, becomes very important.

The aim of the present research was to obtain novel information regarding the nature of MRPs formed during *in vitro* oral gastrointestinal digestion (Hollebeeck, Borlon, Schneider, Larondelle, & Rogez, 2013). Simplified meal systems comprising an average meal composed of bovine serum albumin (BSA), digestible starch and oil; as well as, sugar-containing meals prepared with BSA and glucose or fructose or HFCS were tested. We further analyzed simple amino acid systems prepared using lysine or arginine and fructose or glucose under compatible intestinal conditions to evaluate their contribution. Analyses of amino acid content, fructosamine, carboxymethyllysine (CML), methylglyoxal (MGO)-derivative AGEs, and fluorescent adducts were performed. This is the first study reporting the formation of MRPs associated to the pathogenesis of diabetes and its complications during digestion of simplified meals.

2. MATERIALS AND METHODS

2.1. Reagents

Alpha-amylase from human saliva (type IX-A), porcine pepsin from gastric mucosa (3.200–4.500 U/mg protein), pancreatin from porcine pancreas, porcine bile extract, BSA, L-lysine, L-arginine, D-(-)-fructose, D-(+)-glucose, soluble starch, cellulose microcrystalline, potassium and sodium chloride, sodium carbonate and bicarbonate, sodium phosphate monobasic and dibasic, 1-deoxy-1-morpholinofructose (DMF), nitroblue tetrazolium (NBT), magnesium chloride, diethanolamine and Tween 20 were from Sigma-Aldrich (St. Louis, MO, USA). Ninhydrin was provided by Biochrom Ldt. (Cambridge, UK). CML standard ((S)-2-amino-6-(carboxymethyl-amino)-hexanoic acid, CAS-No. 5746-04-3) was purchased from Iris Biotech GmbH (Marktredwitz, Germany). Competitive ELISA, based on monoclonal antibodies for CML (6C7) and MGO-derivative AGEs (3D11 mAb) (Cai et al., 2002) were used to detect these relevant AGEs. The test sensitivity for CML and MGO-derivatives AGEs was 0.1 U/ml and 0.004 nmol/ml, respectively; the intra-assay variation was ±2.6% (CML) and ±2.8% (MGO-derivatives AGEs) and the inter-assay variation was ±4.1% (CML) and ±5.2% (MGO-

derivatives AGEs). The AGE content of each sample was based on the mean value of at least three measurements per sample and expressed as µg eq./ml. The secondary antibody (goat anti-mouse IgG (H+L)) was obtained from MP Biomedicals (Solon, OH, USA) and the alkaline phosphatase substrate (4-nitrophenyl phosphate disodium salt hexahydrate) from Sigma Aldrich (St. Louis, MO, USA). The blocking buffer (SuperBlock™ (PBS)) was purchased from Thermo Fisher Scientific Inc. (USA). Water was purified using Milli-Q and Elix system. All other chemicals and reagents were of analytical grade.

2.2. Preparation of the simplified meal systems

-An average meal was prepared with BSA (0.18 mM), soluble starch (56 mg/ml) and sunflower oil (8.3 mg/ml).
-Simple sugar-containing meals were prepared as follows: 1) BSA (0.18 mM) and glucose (43 mM); 2) BSA (0.18 mM) and fructose (43 mM); and 3) BSA (0.18 mM) and HFCS (43 mM, 60% fructose and 40% glucose).

These samples were based on the main macronutrients of a meal (carbohydrates, protein and oil). Non-reactive cellulose powder was incorporated to adjust all of them to the same final weight. The concentrations were selected according to the volumes of ingested and secreted fluids, as well as the relative proportions of the dietary macronutrients. For a standard meal, we assumed an intake of 120 g of total carbohydrates, 25 g of protein and 25 g of lipids. In 24 h, a human drinks roughly 2 l, and secretes 1 l of saliva, 2 l of gastric juice, 1 l of bile, 2 l of pancreatic juice, and 1 l of intestinal juice. Considering an average of three meals per day, the physiological concentration of each nutrient was calculated in the gastrointestinal tract (Hollebeeck et al., 2013; Tortora & Grabowski, 1996).

These simplified meals were then digested in duplicate under *in vitro* oral gastrointestinal human digestion conditions (Hollebeeck et al., 2013) with slight modifications, in order to evaluate the formation of MRPs during the digestive process. Briefly, all three stages, salivary (pH 6.9, 10 ml, 5 min, 3.9 U α-amylase/ml, aerobic), gastric (pH 2, 13 ml, 90 min, 71.2 U pepsin/ml, aerobic), and abiotic duodenal step (pH 7, 16 ml, 150 min, 9.2 mg pancreatin, aerobic) were performed in the same flask at 37 ºC. BSA (0.18 mM) was included as the control. A complete digestion of the starch during the digestive process of the average meal could yield up to 314 mM glucose equivalents. The resulting digests were then centrifuged and the soluble fractions were frozen at -20°C, lyophilized (Telstar Lyobeta-15 lyophilizer (Telstar, Spain)) and stored at room temperature until further analysis.

2.3. Preparation of simple amino acid systems

In order to gain insight into the contribution of the single amino acids (lysine and arginine) on the MRP formation during the digestion process of the above described meals, simple systems constituted by basic amino acids (40 mM) and sugars (glucose or fructose) at two different sugar concentrations were prepared as follows: 1) lysine (40 mM); 2) lysine (40 mM) and glucose (43 mM); 3) lysine (40 mM) and glucose (314 mM); 4) lysine (40 mM) and fructose (43 mM); 5) lysine (40 mM) and fructose (314 mM); 6) arginine (40 mM); 7) arginine (40 mM) and glucose (43 mM); 8) arginine (40 mM) and glucose (314 mM); 9) arginine (40 mM) and fructose (43 mM); 10) arginine (40 mM) and fructose (314 mM). These systems were then incubated mimicking lumen intestinal conditions *in vitro* (pH 7, 37 ºC, 2h 30 min).

Simple amino acid systems prepared with lower sugar concentration (43 mM): corresponds to physiological concentrations likely to be present in the lumen of the intestine when ingesting simple sugar-containing meals or ingesting HFCS beverages.

Simple amino acid systems prepared with higher sugar concentration (314 mM): corresponds to a theoretical maximal concentration present as the result of complete digestion of the average meal containing starch. This sugar concentration does not prevail in the lumen under physiological conditions. However, such amount of sugar could be release during the digestion of average meal. As a consequence, it is interesting to study these theoretical sugar digestion conditions to identify the maximal amount of MRPs that can be formed from the precursors without taking into account the rate of sugar absorption. The information to be obtained from such model is very relevant from a food chemistry point of view. The model provides information regarding the reactivity of the different amino acids composing the food proteins and the maximal large number of MRPs that can be generated in the gut during the digestion process. There is a lack of information on this completely new field, which is of great interest because of the impact of MRPs and essential amino acids in human health.

A delay on the absorption of the sugar due to either fructose malabsorption or change in intestinal membrane functionality because of the presence of fat has been assumed either for simplified meal and amino acids systems. Fat present in simplified meal also may be a substrate of the MR causing the formation of non-fluorescence adducts such as CML. Theoretically, intestinal physiological (≤ 50 mM) and non-physiological conditions (50-100 mM) regarding the glucose or fructose concentrations (Ferraris, Yasharpour, Lloyd, Mirzayan, & Diamond, 1990) can be achieved in both cases avoiding clinically evident diarrhea.

In addition, a kinetic study was performed on the system constituted by lysine (40 mM) and fructose (43 mM) in order find out how much time of reaction is necessary for the formation of MRP. This system was incubated under lumen intestinal conditions *in vitro* (pH 7, 37 ºC) for 1h, 2h, and 3h. Samples in duplicate were then lyophilized (Telstar Lyobeta-15 lyophilizer (Telstar, Spain)) and stored at room temperature until further analysis.

2.4. Amino acid content analysis

Amino acid analysis was performed directly on digests from the simplified meals, prepared as indicated in section 2.2. The analysis was carried out using an amino acid analyzer (Biochrom 30, Biochrom Ldt. Cambridge, UK). It is based on an acid hydrolysis of the sample followed by cationic exchange chromatography (high pressure PEEK column packed with Ultropac 8 cation exchange resin sodium form) with post column derivatization using ninhydrin. Samples (1 ml) were first hydrolyzed with 3 ml of HCl (10.6 N). The hydrolysates were then filtered through grade filter paper, and filtrate (0.5 ml) was applied to a SepPak® C18 cartridge (Waters Cromatografía, S.A., Barcelona, Spain) previously activated with methanol (5 ml) and water (10 ml). Finally, samples were eluted with 3 ml of HCl (3 N), and 70 µl of this volume were injected in the chromatograph. Analysis was performed in duplicate and results were expressed as mmol/g BSA.

2.5. Early glycation products

The formation of fructosamine was measured on both simplified meals and amino acid systems.

2.5.1. Fructosamine assay

NBT assay was carried out to determine fructosamine following the micromethod of Vlassopoulos, Lean, & Combet (2013) in both the simplified food models and the amino acid systems. Briefly, samples (25 µl) were mixed with 100 µl nitroblue tetrazolium (0.25 mM) previously dissolved in sodium carbonate buffer (100 mM, pH 10.8). Microplates were incubated for 20 min at 37 ºC and measured spectrophotometrically (BioTek powerWaveTM XS microplate spectrometer (BioTek Instruments, U.S.A)) against control at 530 nm. The fructosamine analogue (DMF) was used as standard (0.5-5 mM). All measurements were performed in triplicate and expressed as µg DMF eq./ml.

2.6. Advanced glycation end products (AGEs)

The analysis of CML and MGO-derivative AGEs, and fluorescent adducts was performed in both the average and sugar-containing meals, and in the amino acid systems.

2.6.1. Carboxymethyl lysine (CML) and MGO-derivative AGEs

Competitive ELISA, based on monoclonal antibodies for CML (6C7) and MGO-derivative AGEs (3D11 mAb) (Cai et al., 2002) was used to detect these relevant AGEs. The test sensitivity for CML and MGO-derivatives AGEs was 0.1 U/mL and 0.004 nmol/ml, respectively; the intra-assay variation was ±2.6% (CML) and ±2.8% (MGO-derivatives AGEs) and the inter-assay variation was ±4.1% (CML) and ±5.2% (MGO-derivatives AGEs). The AGE content of each sample was based on the mean value of at least three measurements per sample and expressed as µg eq./ml.

CML content was also quantitated by using an amino acid analyzer (Biochrom 30, Biochrom Ldt. Cambridge, UK). It comprises an ion exchange HPLC column (high pressure PEEK column packed with Ultropac 8 cation exchange resin sodium form) with post column derivatization using ninhydrin Quantitation was carried out according to the external standard method using a commercial standard of pure CML. Results were expressed as µM.

2.6.2. Fluorescent adducts

Fluorescent adducts were measured by fluorescence spectrophotometry (microplate fluorescence reader, FP-6200 (JASCO, Easton, U.S.A)) using 360 ± 40 nm and 460 ± 40 nm as excitation and emission wavelengths, respectively. Samples (150 µl) were added to black microplates in triplicate and results were expressed as relative fluorescence units (RFU).

3. RESULTS

3.1. Progression of the MR during digestion of food simplified meals

3.1.1. Amino acid content as first indicator

Figure 1 illustrates the consumption of the basic amino acids, lysine and arginine, during the digestive process of an average meal and sugar-containing meals. As can be observed, a significant decrease ($p < 0.05$) of both amino acids occurs for all the tested meals. The arginine amino acid presented a decrease 2 fold greater than lysine (24% vs 11.70%). The meal containing HFCS and the average meal with starch and oil demonstrated significantly ($p < 0.05$) higher loss of these amino acids (34.05% lysine and 35.4% arginine; and 23.44% lysine and 32.7% arginine, respectively) than the glucose and fructose-containing meals (12.20% lysine and 23.92% arginine; and 11.70% lysine and 24.51% arginine, respectively). In the case of arginine content, the average meal and the sugar-containing meals with HFCS presented amino acid reduction during digestion of the same order of magnitude ($p > 0.05$). Furthermore, significant ($p < 0.05$) consumption was observed for other amino

acids different from lysine and arginine released from the BSA during the digestive process (table 1). Glycine (42.88-100% blocked), serine (27.78-100% blocked), tyrosine (42.49-54.05% blocked), isoleucine (34.22-45.07% blocked) and phenylalanine (19.93-54.08% blocked) exhibited the greatest reactivity for all the meals tested. Indeed, the average meal showed complete blockage of several amino acids (aspartic, threonine, serine, glutamic, glycine, alanine, cysteine and valine) released from the BSA during the digestion process. Both, the meal composed of BSA and HFCS and the average meal significantly exhibited the largest total amino acid loss ($p < 0.05$) among all the meals.

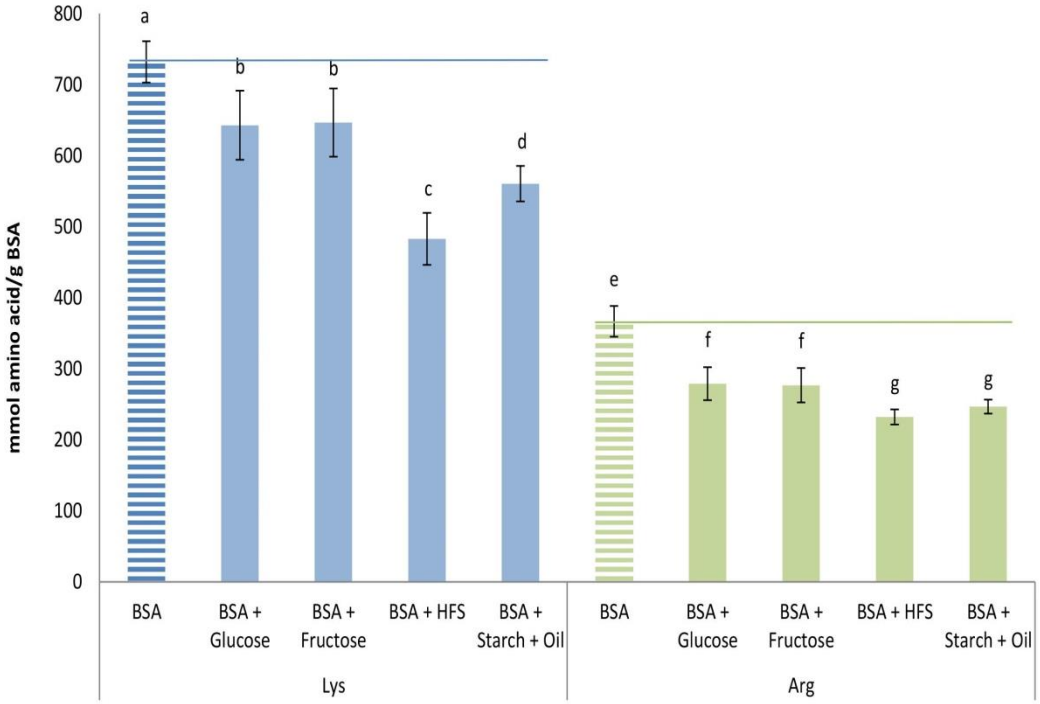

Figure 1. Amino acid content (lysine (lys) and arginine (arg)) of an average meal [BSA (0.2 mM) + starch (≈314 mM glucose) + oil] and high-sugar meals [BSA (0.2 mM) + glucose (43 mM); BSA (0.2 mM) + fructose (43 mM); BSA (0.2 mM) + high fructose corn syrup (HFCS) (43 mM sugars, 60% fructose : 40% glucose] submitted to *in vitro* simulated oral-gastrointestinal digestion. Data are presented as mean (n = 6) ± standard deviation. Different letters indicate significant differences ($p < 0.05$) between samples.

Table 1. Content of other amino acids (AAs) and their percentage of blockage in an average meal [BSA (0.2 mM) + starch (≈314 mM glucose) + oil] and sugar-containing meals [BSA (0.2 mM) + glucose (43 mM); BSA (0.2 mM) + fructose (43 mM); BSA (0.2 mM) + high fructose corn syrup (HFCS) (43 mM sugars, 60% fructose : 40% glucose] submitted to *in vitro* simulated oral-gastrointestinal digestion.

		Sugar-containing meals						Average meal	
	BSA (control)	BSA + Glucose		BSA + Fructose		BSA + HFCS		BSA + Starch + Oil	
Other AAs	**µmol/g BSA**	**µmol/g BSA**	**% blocked**	**µmol/g BSA**	**% blocked**	**µmol/g BSA**	**% blocked**	**µmol/g BSA**	**% blocked**
Aspartic	890.05±35.6^{a}	682.2±54.58^{b}	23.35	690.64±53.1^{b}	22.40	641.7±0.6^{b}	27.90	N.D.	100
Threonine	503.46±23.07^{a}	407.97±32.73^{b}	18.97	415.7±33.71^{b}	17.43	377.17±7.56^{b}	25.08	N.D.	100
Serine	450.02±19.89^{a}	324±23.39^{b}	27.78	334.84±28.39^{b}	25.59	292.37±6.56^{b}	35.03	N.D.	100
Glutamic	1112.25±42.76^{a}	929.92±71.96^{b}	16.39	942.36±70.7^{b}	15.27	858.69±10.74^{b}	22.80	N.D.	100
Glycine	460±16.11^{a}	258.69±21.7^{b}	43.76	262.77±23.49^{b}	42.88	235.27±0.99^{b}	48.85	N.D.	100
Alanine	684.59±29.43^{a}	556.97±46.25^{b}	18.64	565.69±44.83^{b}	17.37	507.68±12.73^{b}	25.84	N.D.	100
Cysteine	171.15±14.12^{a}	153.92±14.33^{a}	10.07	149.63±12.57^{a}	12.57	138.66±4.38^{a}	18.98	N.D.	100
Valine	573.35±17.3^{a}	457.33±34.41^{b}	20.24	466.4±24.65^{b}	18.65	425.41±21.28^{b}	25.80	N.D.	100
Methione	21.24±1.79^{a}	11.74±1.44^{b}	44.70	31.36±1.86^{c}	0.00^{c}	41.06±5.62^{d}	0.00	65.53±4.18^{e}	0.00
Isoleucine	236.54±12.34^{a}	150.26±6.33^{b}	36.47	155.61±6.81^{b}	34.22^{b}	129.94±11.26^{c}	45.07	134.54±6.69^{c}	43.12
Leucine	636.92±30.03^{a}	521.32±15.22^{b,c}	18.15	543.89±22.01^{b}	14.61^{b}	486.72±13.19^{c}	23.58^{c}	551.76±49.56^{b}	13.37
Tyrosine	154.55±13.33^{a}	71.02±7.71^{b}	54.05	81.21±7.81^{b,c}	47.45	88.88±2.3^{c}	42.49	75.57±3.28^{b}	51.11
Phenylalanine	86.07±12.33^{a}	39.52±4.76^{b}	54.08	46.34±5.07^{b}	46.16	68.91±7.39^{c}	19.93	63.47±5.97^{c}	26.25
Histidine	237.95±13.13^{a}	197.45±17.29^{b}	17.02	199.48±17.57^{b}	16.16	167.28±12.56^{c}	29.70	171.08±6.24^{c}	28.10
Total AAs	7320±310^{a}	5460±470^{b}	**25.41**	5690±220^{b}	**22.24**	5280±110^{b}	**27.88**	1900±70^{c}	**74.06**
% Lys+Arg of the total blocked		9.57%		10.87%		17.15%		5.35%	

N.D. Not Detected. Data are presented as mean ± standard deviation; duplicate of sample preparation and duplicate of analysis (n = 6). Different letters indicate significant differences ($p < 0.05$) between the samples of the same row.

3.1.2. Early MRPs and AGEs

The formation of MRPs during the *in vitro* simulated oral-gastrointestinal digestion of the average meal and sugar-containing meals is shown in table 2. Regarding the MRPs produced in the early stages of the reaction, fructosamine was detected. This compound was significantly ($p < 0.05$) found, in descending order, in the average meal, HFCS- and glucose-containing meals. Fructosamine was not detected in the meal prepared with BSA and fructose. On the other hand, the analyses of the advanced MRPs showed the presence of CML and MGO-derivatives AGEs determined by ELISA, and fluorescent adducts by fluorescent measurement, differed among the samples (table 2). The meal constituted by BSA and fructose was the only one that presented significant ($p < 0.05$) formation of CML. Of interest, the meal with BSA and HFCS did not showed formation of CML despite the presence of fructose. However, MGO-derivatives AGEs were significantly ($p < 0.05$) detected in both glucose- and fructose-containing meals but no significant ($p > 0.05$) differences were found between these samples. Again, the meal with BSA and HFCS did not show MGO-derivatives AGEs despite the presence of both fructose and glucose. Furthermore, fluorescents MRPs during the digestive process were detected only in the meal composed of BSA, starch and oil

(average meal), and that prepared with BSA and HFCS. The average meal exhibited the highest amount of fluorescents adducts.

Table 2. Formation of Maillard reaction products (MRPs) during *in vitro* simulated oral-gastrointestinal digestion of an average meal [BSA (0.2 mM) + starch (≈314 mM glucose) + oil] and high-sugar meals [BSA (0.2 mM) + glucose (43 mM); BSA (0.2 mM) + fructose (43 mM); BSA (0.2 mM) + high fructose corn syrup (HFCS) (43 mM sugars, 60% fructose : 40% glucose].

		Early MRPs	Advanced MRPs		
		Fructosamine (µg DMF eq./ml)	CML (µg eq./ml)	MGO-derivative AGEs (µg eq./ml)	Fluorescent adducts (RFU)
	BSA	N.D.	3.91 ± 0.90^{a}	10.22 ± 1.57^{d}	1316.17 ± 54.71^{a}
Sugar-containing meals	BSA + Glucose	29.64 ± 1.05^{a}	4.12 ± 0.56^{a}	12.20 ± 1.63^{a}	1387.17 ± 75.96^{a}
	BSA + Fructose	N.D.	5.03 ± 1.09^{b}	12.23 ± 1.50^{a}	1039.78 ± 52.68^{b}
	BSA + HFCS	42.57 ± 4.70^{b}	1.63 ± 0.37^{c}	8.96 ± 1.27^{b,d}	2270.17 ± 119.55^{c}
Average meal	BSA + Starch + Oil	332.89 ± 10.37^{c}	3.17 ± 1.02^{c}	7.16 ± 1.11^{b}	9282.50 ± 188.33^{d}

N.D. Not Detected. Data are presented as mean ± standard deviation; duplicate of sample preparation and triplicate of analysis (n = 6). Different letters indicate significant differences ($p < 0.05$) between the samples of the same column.

3.2. Progression of the MR in simple amino acid systems

3.2.1. Early MRPs and non-fluorescent AGEs

In order to better understand the formation of MRPs during the human digestion, simple systems constituted by lysine or arginine and glucose or fructose were studied. Table 3 presents the contribution of these amino acids and sugars to the intestinal MRPs' formation. Fructosamine was detected only in those systems containing high sugar concentrations (314 mM). The presence of fructose significantly ($p < 0.05$) caused greater formation of fructosamine (3 and 6 times more, for lysine and arginine systems, respectively) than glucose suggesting that our assay can also detect Heyns products. Lysine system presented higher amount of fructosamine than arginine ($p < 0.05$) when incubated with glucose (314 mM). Fructosamine was of the same order of magnitude ($p > 0.05$) for both amino acids in presence of fructose (314 mM). Fructosamine was not detected in any of the systems with physiologiccal concentration of either glucose or fructose (43 mM).

In the analysis of advanced MRPs measured by ELISA (table 3), the presence of fructose led to a significantly ($p < 0.05$) greater amount of these advanced compounds than glucose. Lysine incubated with 43 mM of fructose showed 14.69±2.19 µg CML eq./ml while 20.21±3.55 µg CML eq./ml ($p < 0.05$) was detected in presence of 314 mM, and both values were significantly higher ($p <$

0.05) than the control (lysine, 11.42±1.84 µg eq./ml). This result was confirmed by the identification of CML by using an amino acid analyzer as described in section 2.6.2. An amount of 1.86 µM for CML was detected in the intestinal lysine and fructose (314 mM) system, and also several unidentified peaks of compounds formed were revealed (chromatograms provided as supplementary material, figure 1S). In addition, it was already observed significant ($p < 0.05$) formation of CML (13.16±1.75 µg/ml) within 1 hour of incubation of lysine and fructose (43 mM) at intestinal conditions (pH 7, 37ºC) compared to the control (lysine, 9.77±1.20 µg/ml). In contrast, MGO-derivatives AGEs were detected only in the arginine systems containing high fructose concentrations (314 mM).

Table 3. Contribution of the basic amino acids, arginine (Arg, 40 mM) and lysine (Lys, 40 mM) to the formation of Maillard reaction products (MRPs) mimicking lumen intestinal conditions *in vitro* (2h 30 min, pH 7, 37 ºC) when incubated with glucose or fructose at 43 or 314 mM under the conditions described in section 2.3.

	Early MRPs	Advanced MRPs
	Fructosamine (µg DMF eq./ml)	MGO-derivative AGEs (µg eq./ml)
Lys	N.D.	5.63±0.17^{a,d}
Lys + Glucose (43 mM)	N.D.	5.75±0.76^{a,d}
Lys + Glucose (314 mM)	51.21±2.53^{a}	4.53±0.47^{b,c,d,e}
Lys + Fructose (43 mM)	N.D.	5.79±0.71^{a}
Lys + Fructose (314 mM)	165.43±5.78^{b}	5.29±0.57^{a,b,d}
Arg	N.D.	4.54±0.24^{b,c,e}
Arg + Glucose (43 mM)	N.D.	3.71±0.53^{c}
Arg + Glucose (314 mM)	30.77±1.24^{c}	5.07±0.56^{d,e}
Arg + Fructose (43 mM)	N.D.	4.44±0.76^{c,e}
Arg + Fructose (314 mM)	178.99±15.00^{b}	18.86±0.97^{f}

N.D. Not Detected. Data are presented as mean ± standard deviation; duplicate of sample preparation and triplicate of analysis (n = 6). Different letters indicate significant differences ($p < 0.05$) between the samples of the same column.

3.2.2. Fluorescent AGEs

The formation of fluorescent adducts in the amino acid systems incubated under intestinal conditions is shown in Figure 2. All systems composed of lysine and glucose or fructose presented significant ($p < 0.05$) formation of fluorescent adducts (≈ 20% increase) but differences were not detected ($p > 0.05$) among them. In the case of the amino acid systems constituted by arginine, a very significantly lower fluorescence ($p < 0.05$) was found for the arginine control compared to lysine control. The formation of fluorescent adducts experimented a 3-fold increase for those arginine systems prepared

with glucose and a 5-fold increase for those prepared with fructose. A dose-response relationship was not detected for any of the systems

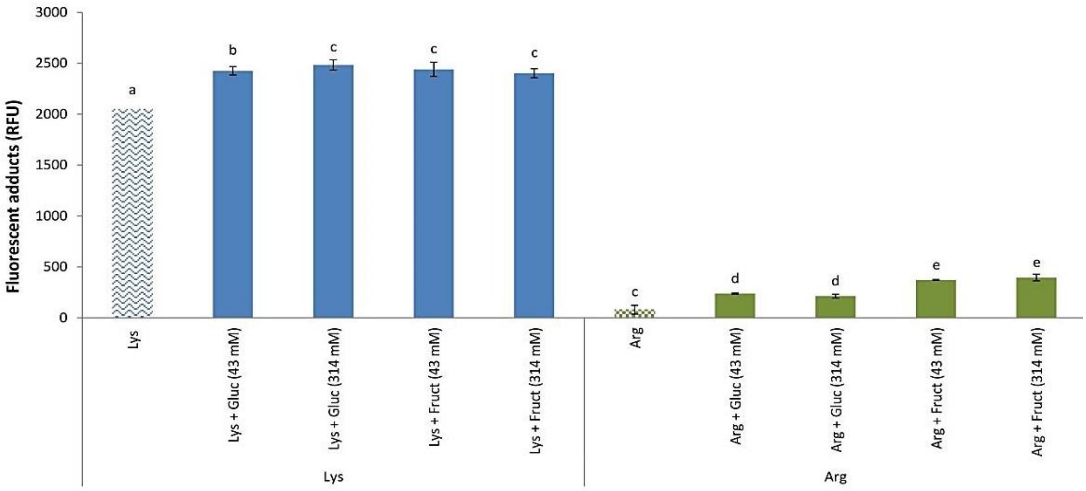

Figure 2. Formation of fluorescent adducts during incubation of basic amino acids (lysine (lys) and arginine (arg), 40 mM) with glucose (gluc) or fructose (fruct) at 43 and 314 mM under lumen intestinal conditions (2h 30 min, pH 7). Data are presented as mean (n = 6) ± standard deviation. Different letters indicate significant differences ($p < 0.05$) between samples.

4. DISCUSSION

The data generated confirm the occurrence of the MR during the digestive process of an average meal (BSA, starch and oil) and sugar-containing meals (BSA and glucose or fructose or HFCS); as well as, during incubation of systems with only a reactive amino acid and fructose within the time period expected for a normal digestion and assuming delay of the sugar absorption due to its malabsorption or fat presence. As expected, the formation of the MRPs differed depending on the nature of the meal.

The loss of free amino acids is the first indicator of the occurrence of the MR during the digestion process of the meals (table 1 and figure 1). During gastrointestinal digestion of dietary proteins (BSA), amino acids and peptides can be released during the gastric and intestinal steps by chemical and enzymatic hydrolysis. The amino groups, from the released amino acids and peptides, are susceptible to react with reactive carbonyl groups present in the intestinal lumen. Therefore, the reaction comprises not only the formation of MRPs from the amino groups of the arginine and lysine side chains, but also the alpha amino groups of all the released amino acids (figure 3). Basic amino acids (arginine, lysine) and others with different polarity (glycine, serine, tyrosine, isoleucine and

phenyl alanine) were highly blocked. These results are in line with data previously reported for amino acid reactivity in the MR at different studied conditions (Golon, Kropf, Vockenroth, & Kuhnert, 2014; Kwak & Lim, 2004; Mennella, Visciano, Napolitano, del Castillo, & Fogliano, 2006; P. Yu, Xu, & Yu, 2016).

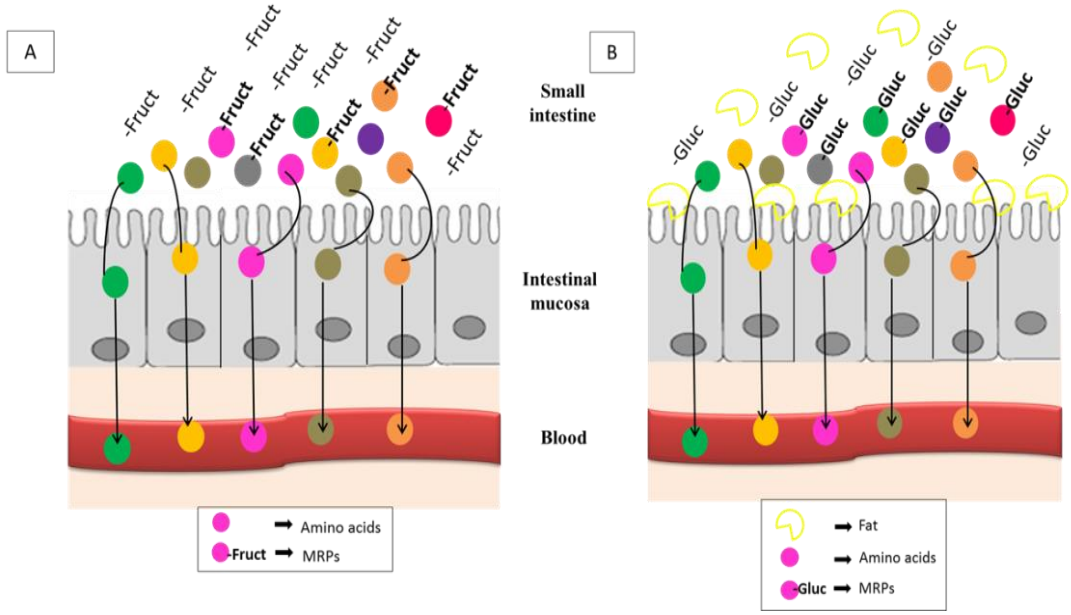

Figure 3. Formation of Maillard reaction products (MRPs) in the intestinal lumen through the reaction between different amino acids released from the BSA protein during the digestive process and (A) fructose at physiological concentrations (43 mM) under malabsorption state and (B) glucose at a maximum theoretical concentration released from the complete digestion of starch of an average meal (314 mM) containing fat which modulates the intestinal epithelial membrane.

Of interest, it has been described that in malabsorption state, the fructose not absorbed in the intestine may react with tryptophan from food through the MR and block its absorption, which causes a potential reduction in the synthesis of serotonin and thereby symptoms of depression (Huether, Kochen, Simat, & Steinhart, 1999; Ledochowski, Widner, Bair, Probst, & Fuchs, 2000). It is described that fructose possess higher proportion of open-chain form compared to glucose and therefore greater active form to react in the MR (Laroque et al., 2008). This may be the reason why HFCS, which presents both sugars combined in a 60:40 ratio (fructose : glucose), achieved higher loss of amino acids than glucose and fructose individually This result might support recent epidemiological investigations describing a relationship between intake of HFCS-containing beverages and development of asthma, bronchitis and arthritis as a consequence of glycation reactions within the lumen of the intestine (DeChristopher et al., 2015a, 2015b, 2016).

Furthermore, the remarkable loss of amino acids in the average meal may be due to its content in starch and oil. The high glucose amount potentially released from the starch (≈314 mM) during the digestion suggests greater formation of MRPs. Likewise, many researchers reported a positive role of dietary lipids promoting the MR by the formation of lipid-derived reactive carbonyls from the lipid oxidation (Hidalgo, León, & Zamora, 2016; L. Yu et al., 2016; Zamora & Hidalgo, 2011). *In vivo* digestion, however, is rapidly followed by absorption leaving little time for the MR unless there is a significant simultaneous sugar malabsorption. The latter is likely during ingestion of large loads of HFCS-rich beverages or foods containing fat.

The simplified pathways for the formation of early and advanced MRPs during the digestive process of the studied meals and amino acid systems are schematically presented in the figure 4. Briefly, MR includes an initial formation of Schiff's base, followed by intermolecular rearrangement and conversion into Amadori/Heyns products. They undergo further processing to form a heterogeneous group of amino acid-bound moieties, such as non-fluorescent adducts (e.g., CML) and cross-linking fluorescent products (e.g., pentosidine), called AGEs. Pathways of AGE formation involve glucose autoxidation through the generation of α-oxoaldehydes, such as MGO, which is a major precursor of AGEs. Of note, during the digestive process different length peptides with different glycation reactivities are also released along with the release of free amino groups.

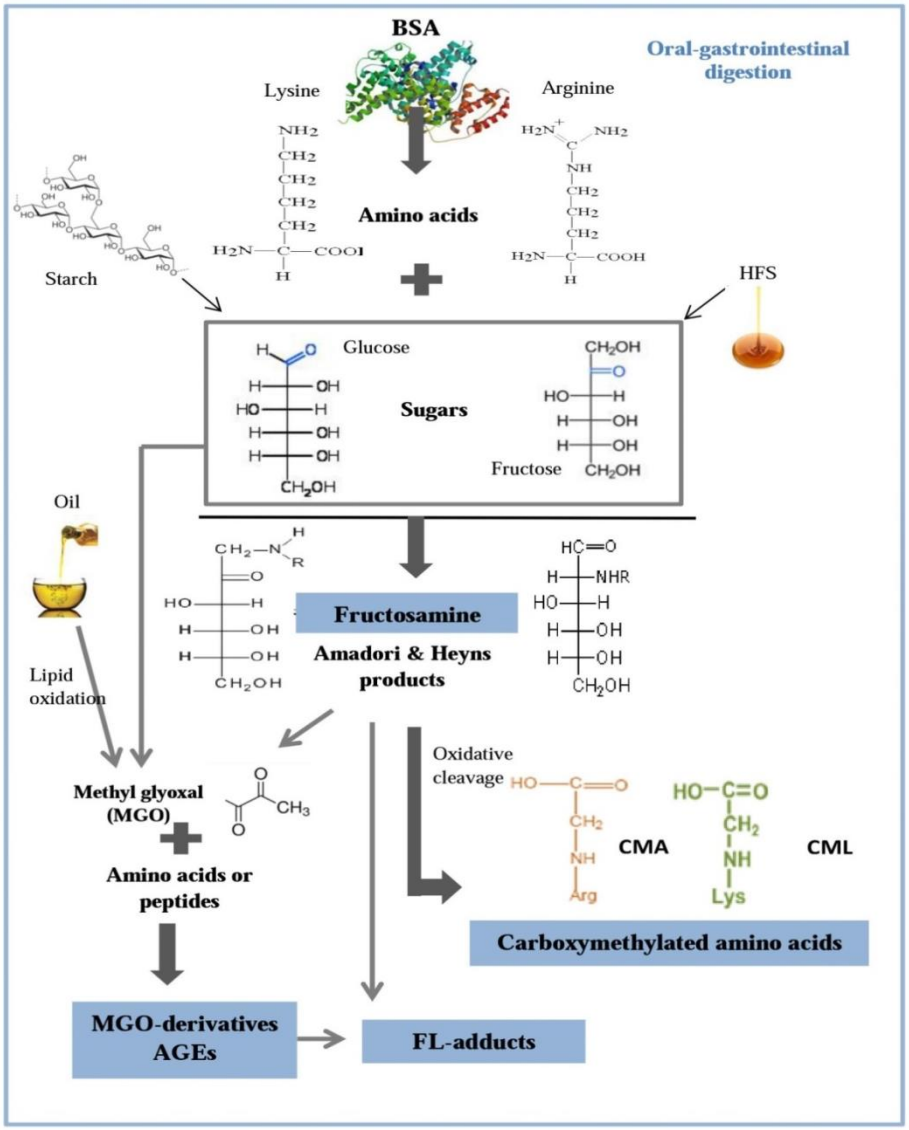

Figure 4. Simplified pathways for the formation of early and advanced Maillard reaction products (MRPs) during the digestive process of an average meal (BSA, starch and oil), high-sugar meals (BSA and glucose or fructose or high fructose corn syrup (HFCS) and simple amino acids systems (lysine or arginine and glucose or fructose), as described in section 2.2 and 2.3

Fructosamine, an early MRP, was found in most meal systems, except in those containing only BSA and fructose. Formation of fluorescent adducts occurred mostly in the meal systems with the highest formation of fructosamine, which suggests pathways going mostly into the formation of cross-linking fluorescent products such as pentosidine rather than CML. The non-fluorescent AGEs (CML and MGO-derivative AGEs) were found exclusively in the meal prepared with BSA and fructose (43 mM)

(table 2) which is supported by the previously commented results. The current results confirm the formation of non-fluorescent products during the human digestive process. To date, there are not studies in this area. New evidence is presented in this study. As regards the fluorescent adducts with characteristic fluorescence of AGEs, 7-fold and 2-fold increases were detected for the meals containing starch and HFCS, respectively, during the digestive process (table 2). These results are supported by the analysis of amino acid systems incubated at conditions similar to those in the intestinal lumen (figure 2). In line with these findings, a very recent study showed formation of fluorescent adducts, when incubating free amino acids (arginine, lysine and glycine, 50mM) with fructose or glucose (50 mM) under conditions compatible with those of the digestive system (Bains & Gugliucci, 2017). Unlike what these authors described, we observed high fluorescence of the lysine solution itself (control). This may be due to the capacity of lysine in high concentrations to form self-assembling aggregates (Homchaudhuri & Swaminathan, 2001). Moreover, we could not demonstrate a dose dependent formation of fluorescent AGEs between fructose or glucose and amino acids. The analysis of fluorescent AGEs by measurement of fluorescence intensity is somehow limited and nonspecific. Pentosidine, crossline and pyrropyridine may be some of the fluorescent cross-linked AGEs formed during digestive process (Nursten, 2005). Pentosidine can be formed by the reaction of lysine and arginine, forming a fluorescent crosslink with any of several carbohydrate precursors including glucose (Hatfield, 2005). The identification of several other unidentified peaks in the lysine and fructose (314 mM) system (supplementary material, figure 1S) suggests the formation of compounds that require further studies.

Our finding on formation of MRPs, both early and advanced, during conditions mimicking the digestive process, including concentration of reactants and time of reaction, supports the concept of intraluminal generation of AGEs as another source of exogenous AGEs (DeChristopher et al., 2016). Currently, most workers in the field have suggested that exogenous AGEs come already preformed in certain AGE-rich food we ingest (Uribarri et al., 2010). The fate of exogenous and intestinally generated AGEs is an area that requires intensive further study. We know that a small percent of the ingested AGEs are absorbed into the circulation becoming incorporated into the body pool of AGEs and therefore contributing to an overall state of chronic oxidative stress and inflammation (Vlassara & Uribarri, 2014) and probably the same happens with the compounds generated intraluminally. The largest amount of unabsorbed AGEs will cotinue its transit into the colon where it can interact with the microbiota, react with AGE receptors (RAGE) within the colonic wall initiating a localized inflammation that may eventually propagate systemically or simply be excreted in the stool.

Several studies have reported human colonic microbiota degrading selected glycated amino acids. Higher degradability of early MRPs than advanced products have been shown due to the their lower chemical stability (Hellwig et al., 2015). Some of these MRPs can play a role in colon toxicology, through increased colonic protein fermentation, and may also act as systemic toxicants and inducers of inflammation (Tuohy et al., 2006). Low-grade intestinal inflammation for example plays a key role in the pathophysiology of irritable bowel syndrome (Sinagra et al., 2016). Moreover, these MRPs can modulate gut microbiota composition (Seiquer, Rubio, Peinado, Delgado-Andrade, & Navarro, 2014; Tuohy et al., 2006) and they may act as a growth substrate for detrimental bacteria such as some Clostridium and Bacteroides species (Mills et al., 2008). A relationship between unabsorbed AGEs and the greater prevalence of allergies/inflammation has recently been postulated (Smith, Masilamani, Li, & Sampson, 2015). Moreover, a common colonic bacteria, E. Coli, produces, accumulates and releases AGEs into the medium (Cohen-Or, Katz, Ron, Shanley, & Wheeler, 2011). Therefore they may adversely alter their colonic microbial composition, potentially enhancing their risk for the development of metabolic diseases such as obesity and type 2 diabetes (Cani, Osto, Geurts, & Everard, 2012). As mentioned above, fructose reaction with tryptophan can prevents its absorption decreasing levels of serotonin and perhaps inducing depression (Huether et al., 1999; Ledochowski et al., 2000). The marked loss of amino acids released from proteins though the MR during digestion demonstrated in our data an interesting but usually forgotten issue, which is the loss of the nutritional value of foods resulting from the MR.

5. CONCLUSIONS

Novel information regarding the nature of MRPs formed during the digestive process of simplified average meal and sugar-containing meals was obtained. Content of different amino acids decreased after digestion. Early MRPs and fluorescent adducts were detected in digests of those meals containing HFCS and starch. CML and MGO-derivatives AGEs were found in the meals composed of fructose. Moreover, fluorescent adducts were detected in the control intestinal systems (amino acids alone). According with the results on simplified amino acids systems nature of both components, amino acids and sugars, has an impact on the formation of specific MRPs. Results on the identity of the MR found in simplified amino acids systems and meal agreed. This is the first study proposing the formation of a non-fluorescent AGE associated to the pathogenesis of diabetes and other intestinal inflammatory diseases during digestion of simplified meals containing fructose. On the other hand, our results support that the bioavailability of other amino acids beside arginine and lysine may be greatly reduced by MR during the digestion process which may affect human health

Acknowledgements: This study was funded by the SUSCOFFEE (AGL2014-57239-R) project and carried out in collaboration with the Division of Nephrology of the Icahn School of Medicine at Mount Sinai Hospital (New York, USA). Martinez-Saez, N. thanks the Autonomous University of Madrid (UAM), Spain, for her FPI-predoc fellowship and the financial support for the short stay at the Icahn School of Medicine at Mount Sinai, Division of Nephrology, New York, USA.

Author's contributions: "del Castillo, M.D. and Uribarri, J. designed the experiments, supervised the investigation and revised the manuscript. Martinez-Saez, N., Fernandez-Gomez, B., and Cain, W. performed the experiments and analyzed the data; Martinez-Saez, N. is the principal author of the investigation since it is part of her PhD thesis supervised by del Castillo, PhD."

Conflicts of Interest: Declare conflicts of interest or state "The authors declare no conflict of interest."

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Supplementary material

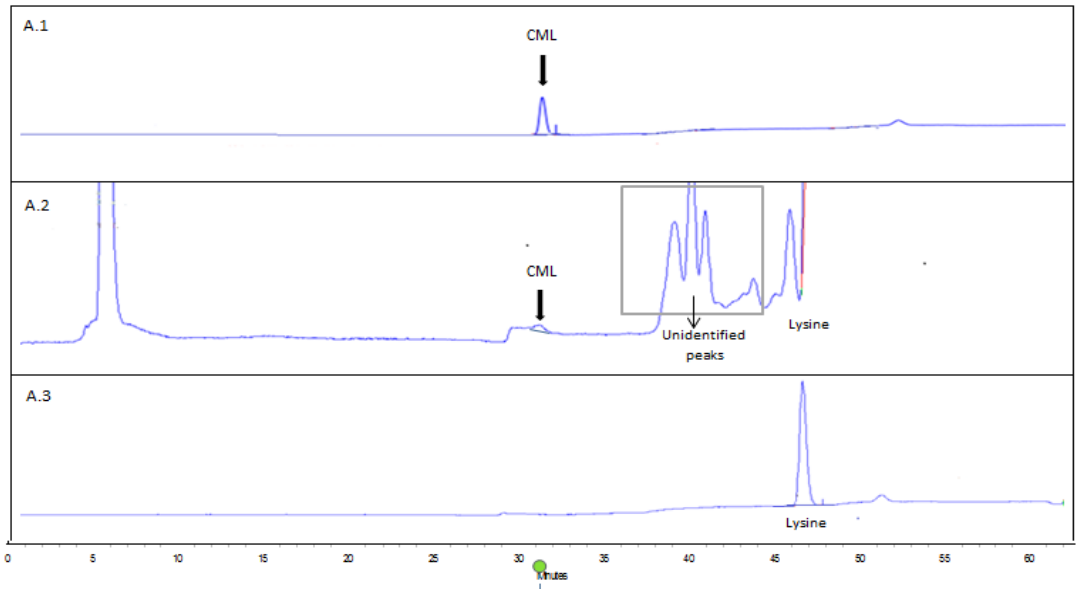

Figure 1S. Identification of carboxymethyl lysine (CML) by amino acid analysis as described in Materials and Methods, in (A.1) a solution of standard CML (5 µg/ml = 26.7µM) and in (A.2) an intestinal system prepared with lysine (40 mM) and fructose (314 mM). CML appeared with a retention time of 31.5 min and unidentified peaks were also present in A.2. Intestinal control of lysine (40 mM) was also included (A.3) which did not show no significant peak other than that of the native amino acid.

General Discussion

GENERAL DISCUSSION

The development of novel foods with health-promoting properties involves several essential steps (***figure 1***). After identifying a potential new bioactive ingredient (step 1) it is necessary to demonstrate its efficacy and to determine the intake level in order to achieve the desired effect (step 2). This efficacious levels should be demonstrated to be safe (step 3). Next, a suitable food vehicle must be developed for the bioactive compounds (foods or beverages) (step 4) and scientific research about their efficacy should be provided (step 5).

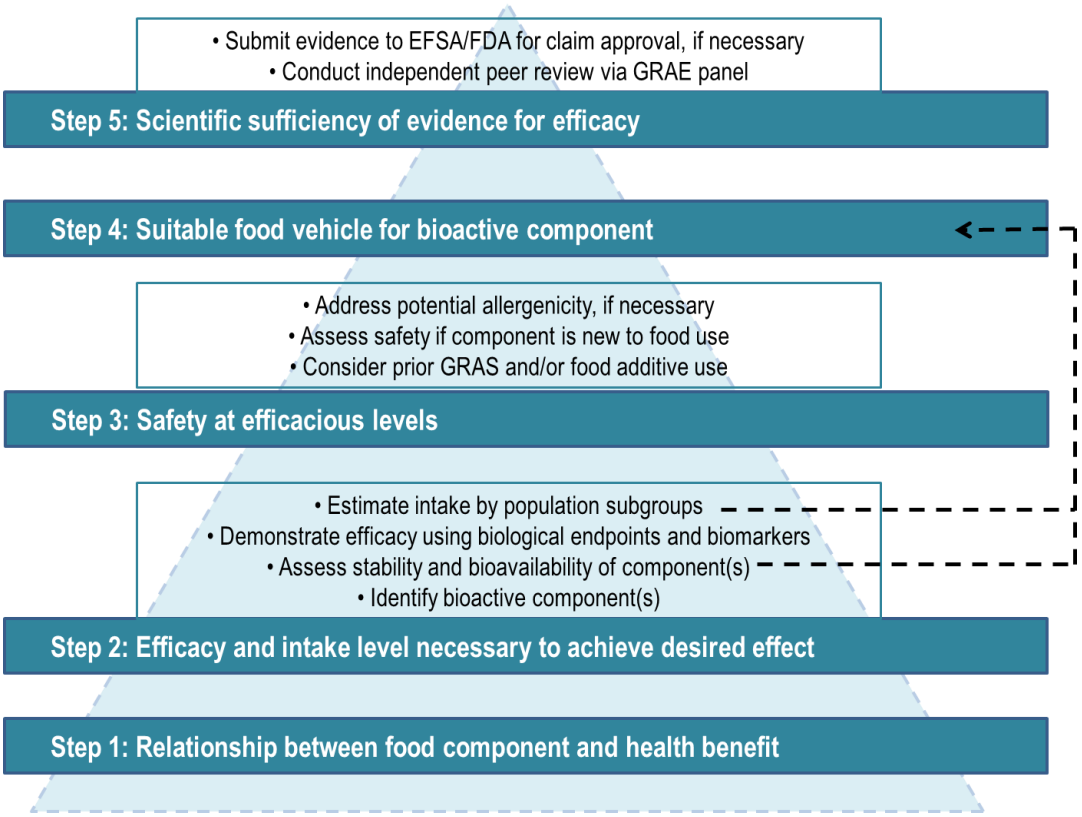

Figure 1. Steps in the development of health-promoting foods. Adapted from Institute of Food Technologists (IFT) (2005) [1].

Sweet drinks, packaged snacks, and biscuits have been shown to contribute significant amounts of energy to the population [2]. As a consequence, interest on consumption of functional foods for reducing the risk of obesity and T2D has significantly increased. In order to satisfy consumers' demands, the present research focuses the attention on the design of sustainable healthier beverages and bakery products. Beverages and bakery products contain coffee by-products, CS and

SCG, as natural sources of health-promoting compounds. Bakery foods were formulated combining coffee components and other food ingredients exerting health-enhanced properties such as non-nutritive sweeteners (stevia) and soluble dietary fibres (FOS).

Several studies have investigated the positive effects of modifying major nutrients of the diet on glycaemia, weight, and other metabolic outcomes. However, the use of bioactive compounds from natural sources as a successful approach in reducing the risk of these metabolic chronic diseases is still a challenge [3].

1. Novel beverages

At present, beverages are by far the most active functional food category because of convenience and possibility to meet consumer demands as well as their excellent delivering means for nutrients and bioactive compounds [4]. The different types of commercially available beverages could be grouped in: (1) dairy-based beverages including probiotics and minerals / ω-3 enriched drinks, (2) vegetable and fruit beverages, and (3) sports and energy drinks. Moreover, the development of novel beverages is focused on the research in the following approaches: (1) exploitation of microorganism functionality, (2) optimization of the production and formulation, (3) use of prebiotics and symbiotics, (4) utilization and processing of natural ingredients and **(5) use of by-products of fruit and food industries as functional ingredients**. This latter approach is the core of the present thesis.

Recovery of bioactive phenols from virgin olive oil wastes and their addition to milk beverages [5], the use of plum skin extract as functional ingredient in fruit juice-based beverages and flavoured iced teas [6] or the production of whey-based fruit beverages [7] are some of the proposed applications to revalorize food wastes. Coffee wastes have been hardly used in the design of functional beverages so far. Qishr is an ancient infusion made from pulp of the fermented coffee berries that today is consumed as a beverage of spiced coffee husks, ginger and cinnamon instead of coffee in Yemen [8]. “Cascara tea” is a fruity taste beverage prepared with the skin of the coffee cherry produced in Central America (http://www.npr.org/sections/thesalt/2015/12/01/456796760/cascara-tea-a-tasty-infusion-made-from-coffee-waste). Moreover, two commercial beverages, based on coffee husks, are attracting the interest of the western consumers. Bai Brands uses coffee husk extract as an ingredient in their beverages, which contain antioxidants and caffeine (http://www.drinkbai.com/), and KonaRed preserves the majority of the nutrients from the coffee plant and the dominant polyphenols (CGA, quinic acid and ferulic acid) in the beverages prepared with coffee husks

(https://www.konared.com/). However, these drinks with coffee by-products have not been associated yet to any particular health benefit beyond those described for their individual bioactive compounds present in the coffee wastes.

The use of CS for the preparation of novel beverages has been proposed for the first time in this study (***chapter 1; figure 2***). The CSE used in this study was obtained by a simple water extraction stage (100 °C for at least 10 min) described in patent WO2013004873 A1. Our previous studies indicated that this green extraction process of CSE produces completely recyclable solid waste enriched in bioactive compounds [9]. Moreover, the present thesis provides knowledge on the potential effect of the CSE prepared as an antioxidant beverage on body fat accumulation *in vivo* and the bioactive compounds that may be responsible of this effect.

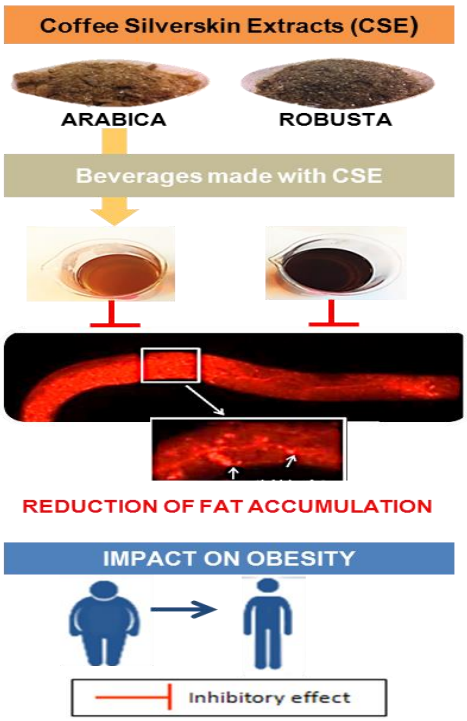

Figure 2. Effect of coffee silverskin extracts (CSEs) on fat accumulation.

The development of novel beverages requires sensorial analyses in order to evaluate the quality attributes of the product and to meet consumers' demands. Coffee beverages are considered to have unique sensory properties. The aroma is the main attribute of the sensory experience for coffee consumers. The brewed coffee sensory attributes are divided in appearance, odour/flavour, taste,

mouthfeel and aftertaste, with multitude of descriptors such as roasty, fruity, rancid, sweet, better and astringent [10]. Our results conclude that the acceptance level of the beverages made with CSE was satisfactory since 95% of the panel were favourable towards the beverages.

The novel beverages based on CSE present reduced sugar content since they were prepared without addition of nutritive sweeteners and glucose amount was not detected. These novel drinks respond to the global obesity concerns and fit perfectly with the requests from the European Union to reformulate drinks and set a target of 10% added sugar reduction. To achieve this goal, food and drink industry are encouraging consumers choices towards low and no calorie drinks. In this sense, the Union of European Soft Drinks Association (UNESDA) has announced a plan to reduce added sugars in all soft drinks by a further 10%, tripling the pace of sugar reduction (http://www.foodnavigator.com/Market-Trends/European-soda-makers-vow-to-reduce-added-sugar-by-further-10). Furthermore, the sugar taxes have being considered by the European governments. Recently, Spain announced plans for a tax on sugar-sweetened beverages (http://www.foodnavigator.com/Policy/Spain-and-Estonia-to-tax-sugary-drinks), which has been into effect by the moment only in Catalonia. The UK government has approved the sugar tax on soft drinks that will come into force April 2018. These sugar taxes could be extended to other foods and drinks products, following research on its effectiveness on reducing obesity after 2 years (http://www.foodmanufacture.co.uk/Regulation/Sugar-tax-on-soft-drinks-may-be-extended-in-UK).

Moreover, regarding the recommendations for a good hydration, the SENC strongly suggests drinking mineral and tap water and sugar-free beverages such coffee and tea as part of a daily intake (***figure 3***) [11]. It is in line with the daily recommendations proposed in the Healthy Eating Plate of Harvard School of Public Health (***figure 5***, page 22).

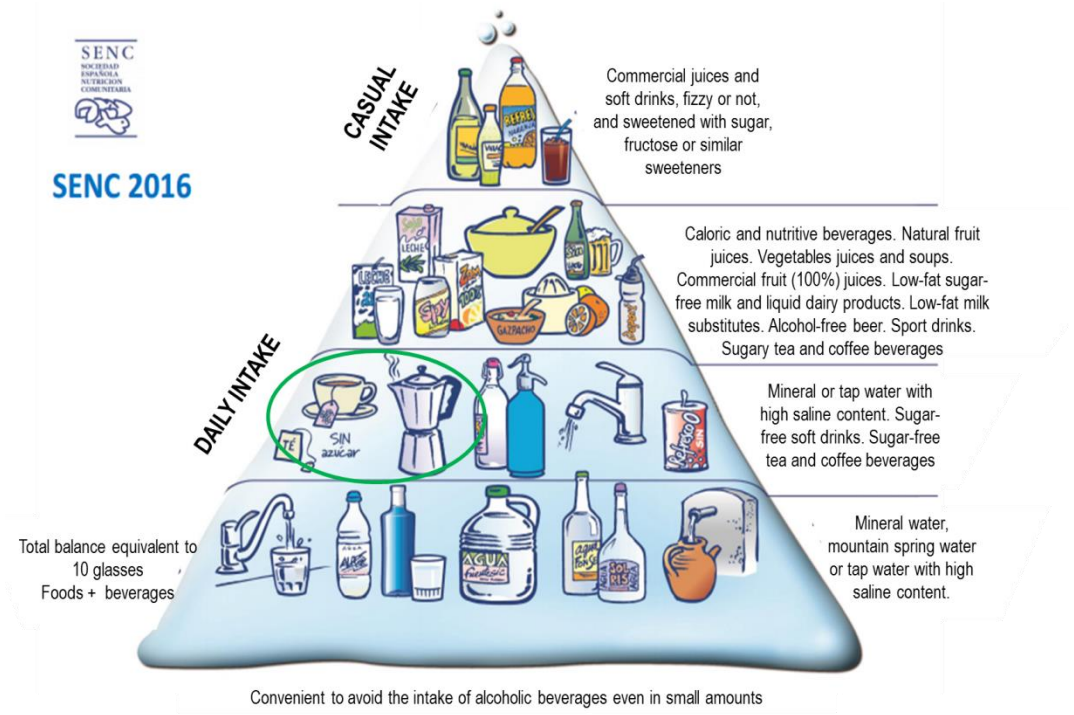

Figure 3. Healthy Hydration Pyramid designed by the Spanish Society of Community Nutrition (SENC, 2016) [11].

Regarding the *in vivo* biological effects of these novel beverages on the body fat reduction, *C. elegans* was employed as the animal model. This worm has been considered an excellent candidate for whole organism-based high-throughput screening in drug assessment as a preclinical model [12], and more recently has become an attractive *in vivo* animal model for initial studies of nutrition interventions prior to confirmation in higher animal species [13]. *C. elegans* conserves 65% of the genes associated with human genes and its intestine presents close similarities to the human gastrointestinal tract [14,15]. Due to the translucent body of these worms the analysis of intestinal fat deposition by lipid-staining dyes is very convenient [16]. Whereas mammals have dedicated adipocytes, *C. elegans* store fat in droplets in their intestinal and hypodermal cells, which can be directly visualized in intact animals [17]. Regarding the lipid metabolism, over 300 genes in *C.elegans* have shown to cause a reduction in body fat when inactivated [16]. Its metabolic pathways have been examined most extensively in the context of insulin signalling since down-regulation of insulin signalling confers an extended adult lifespan and increased fat accumulation. Activation of signalling by the DAF-2 insulin receptor inhibits the FOXO-transcription factor DAF-16, thereby promoting an increased fast metabolism. In contrast, a down regulation of DAF-2 allows activation of DAF-16, which promotes fat accumulation [17]. In addition, a number of enzymes

involved in lipid metabolism are highly expressed in the intestine [18]. The T21H3.1, Y49E10.16, T10B5.7 and F28H7.3 genes have been found to encode lipase enzymes. *C. elegans* releases eight member of the α/β hydrolase lipase family, which are secreted into the intestinal lumen and function as digestive enzymes [19]. They are very similar to the mammalian gastric and pancreatic lipases from the α/β hydrolase lipase family [20].

Our results show that the main bioactive compounds of the CSE, CGA and caffeine, significantly reduce lipid deposits in *C. elegans*. They were found in physiologically active doses in the beverages prepared with CSE, however unexpectedly a synergic and/or additive effect was not detected. Melanoidins, which are also present in the CSE beverages, are constituted by carbohydrates, including dietary fibre (soluble Maillardized fibre), proteins and polyphenols such as CGA [21]. All of them conform the matrix of the beverage and thereby may be affecting the bioaccesibility and bioavailability of CGA and caffeine, respectively, by adhering them to their complex structure [22,23]. Moreover, unlike those beverages prepared with ACSE, those containing RCSE achieved higher effects on lipid metabolism and similar to those found in a commercial dietary supplement, made from Robusta decaffeinated green coffee extract. Robusta specie has been shown to possess higher content of bioactive compounds and CGA appears as the main one to affect lipid metabolism.

Recently, our group has demonstrated that CSE and CGA are able to significantly inhibit the activity of pancreatic lipase *in vitro* [24], which is in charge of the hydrolysis of most of the dietary lipids we intake, and thereby plays a key role in the efficient digestion of triglycerides [25]. Therefore, its inhibition may be one of the possible mechanisms of action exerted by the CSE beverages to reduce fat accumulation in *C.elegans*. Other authors have reported that mulberry leaf polyphenols containing CGA as main polyphenol compound reduced fatty acid storage in *C. elegans*. Subsequent genetic screens and gene expression analyses demonstrated that polyphenol function mainly depended on the germline signalling pathway, thus influencing the activities of down-stream transcription factors, such as DAF-12, DAF-16, PHA-4, and NHR-80; as well as the expression levels of their target genes [26]. Moreover, dietary fibre of the beverages from CSE might also have impact on body fat accumulation. Several studies assayed the effect of supplementation with dietary fibre from barley and oat (0.5, 1 and 3%) in *C.elegans* wild type N2. Both types of dietary fibre showed to reduce body fat, which appeared to be primarily mediated via SIR-2.1, DAF-16, and DAF-16/DAF-2 and DAF-2 gen, respectively [13,27]. All together may be supporting the lyporegulatory character of CSE.

In addition, these novel beverages based on CSE have antioxidant properties, which may be attributed not only to polyphenols, such as CGA, but to other compounds like melanoidins. These

bioactive compounds present multiple biological activities that may enhance the value of these beverages [28]. Nevertheless, further research should be conducted to elucidate the contribution of the different bioactive compounds present in the beverage and their mechanisms of action.

Therefore, it has been achieved a sustainable use of coffee wastes, in particular CSE, in the development of novel beverages for body weight control that also meet European requests on the sugar content reduction and the current consumer's demands.

2. Novel bakery foods

Biscuits represent the largest category of snack item among bakery products [29]. It has become one of the popular snack foods for both young and elderly people due to their affordable price, convenience, shelf-stable, and nutritive value [30]. Biscuits offer a valuable supplementation vehicle for nutritional improvement and bioactive compounds [31]. Components of coffee by-products, such as fibre and non-nutritive compounds, are excellent ingredients to be incorporated in bakery products, in order to enhance their sensorial and nutritional quality, and to simultaneously achieve a sustainable effect in the coffee sector.

Coffee flour (www.coffeeflour.com) is a new ingredient developed from the coffee pulp, presenting high content in fibre and ash, and low fat level [32]. It has been proposed for its utilization in different food formulations such as breads, cookies and muffins with better nutritional properties. The high amount of dietary fibre in coffee husk appears as an advantage in making "energy bars", by grinding the whole coffee husk and thereby, including all antioxidants and fibre into the product. Then, the coffee husk could be launched as an allergic-friendly ingredient since it is naturally gluten free [33]. Just one study on the use of chemically pre-treated CS, as an ingredient of bread formulations, has been reported [34]. However, no previous works on the use of natural whole SCG and CS, as food ingredient for improving technological and nutritional quality of bakery products, have been conducted.

2.1. Bakery products based on coffee silverskin

The present thesis shows the feasibility and effectiveness of applying 1) CS, 2) CSE, or 3) CSE combined with the solid residue recovered from the extraction process, to non-added sugar biscuits where sugar content has been replaced with stevia (***chapter 2, article 1; figure 4***).

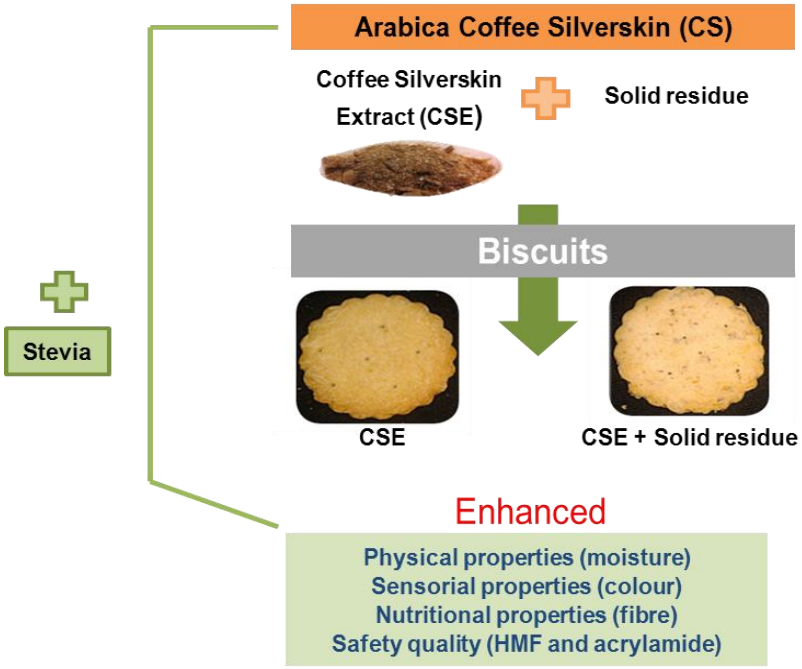

Figure 4. Use of coffee silverskin (CS), CS extract (CSE) and its solid residue in non-added sugar biscuits.

This is the first time it is achieved a full recovery of CS through the whole conversion into two products, natural colouring and source of fibre. On one hand, the content of dietary fibre (6.6% soluble and 60.5% insoluble in CS) [35], plays a key role on the technological quality, as a texturizing agent, and on the nutritional properties, as enhancer of the nutritional value of the biscuits. On the other hand, the colouring has an essential impact on the sensorial properties of the biscuits, which will provide the typical golden colour expected of this type of baked products. This double effect of the use of whole CS on sensory and nutritional quality is essential for achieving high acceptance by the consumers.

MR is one of the prevalent reactions during coffee roasting as well as during biscuit baking process; however, other non-enzymatic food-browning reactions might contribute to the dark colour of the melanoidins formed in the MR (17% in ACSE [36]). The non-enzymatic oxidation of phenols was reported to yield brown coloured structures [37]. The ACSE used in this study as food ingredient possess 1.1% of CGAs [36]. Part of these CGAs might be incorporated via the phenolic acid moiety through non-ester bonds to coffee melanoidin structures, therefore providing the colour of the baked biscuits caused by the oxidation of CGA [38]. Moreover, CS has been shown to possess 1.6-12% of sugar content [39], which may be contributing to brown-coloured compounds formed because of

reaction of caramelization. However, MR yields much more intense colours than caramelization [40] since this latter involves long lengths of time [41], and amino groups and phenolic compounds compete with monomeric sugar-sugar caramelization reactions. Our results showed that sugar-free biscuits have the non–enzymatic browning reactions limited [42]. However, those biscuits containing stevia as non-nutritive sweetener and CSE, did not present significant differences in colour compared with biscuits prepared with sucrose. The results support the validity of using CSE as a natural colouring.

Furthermore, along with the desired flavour and colour-related substances, neo-contaminants, such as acrylamide and HMF, are formed during the MR. Both compounds possess genotoxic and carcinogenic properties [43]. CSE and its main phenolic compound –CGA-, have been shown to exert antiglycative properties, thereby limiting the MR [31,32]. The use of CS as an ingredient of the biscuits, partially improves to a great extent this neo-contaminant formation. In addition, the non-nutritive sweetener –stevia-, plays a key role on the safety quality of the products. Our results show a reduced formation of these neo-contaminants when sugar is replaced with stevia. The combination of both, coffee by-product and non-nutritive sugar replacers, provide a safer product.

Likewise, the digestive process plays a decisive role on the release of compounds of interest in health and disease [44]. The nature and amount of food components, such as carbohydrates, fat, protein, and dietary fibre; as well as, the processing and preparation of the food might influence the release of neo-contaminants from the food matrix [45]. Our results show that after *in vitro* simulated oral-gastrointestinal digestion of the biscuits formulations [46], acrylamide was not bioaccessible to be absorbed. Other authors have also reported a gradual decrease in acrylamide levels through the gastric, duodenal and colon phases during *in vitro* digestion of biscuits [47]. This is due to the Michael addition of amino acids to acrylamide during the digestive process. Acrylamide possess the potential to react with the nucleophilic groups (–SH, $–NH_2$) of amino acid side chains. In fact, cysteine becomes highly reactive toward acrylamide, especially under the simulated duodenal conditions. Therefore, the levels of acrylamide ingested with foods may not directly indicate its absorption rate through gastric, duodenal and colonic routes. Gastrointestinal conditions and the ingested food composition affect the levels of bioavailable acrylamide, and it should be taken into consideration.

Furthermore, these biscuit formulations present low content of bioaccessible glucose, which is in concordance with the European recommendations regarding the formulation of food products with reduced sugar amount. Consequently, these innovative foods may be potentially suitable for diabetics or people who want to lose weight. Further studies should be required to evaluate the

biological effects of these biscuit formulations on the management of these metabolic chronic diseases.

2.2. Bakery products based on spent coffee grounds

For the first time, it has been proposed the use of SCG from the industrial instant coffee process as food ingredient in the development of a healthy product line of pastry, confectionery and/or bakery products such as bread, buns, biscuits, breakfast cereals and snacks, among others (***patent WO2014128320 A1; figure 5; Annex 1***) [48]. The patented application proposes the utilization of SCG as sustainable natural antioxidant insoluble dietary fibre. Since SCG come from the food industry, their use as coffee fibre is of food grade as well, and its conversion in a co-product may be feasible, regarding food safety and traceability. It can be used in the range between 4-8%, which corresponds to the nutrition claims "source of fibre" and "high fibre content", respectively (European Regulation (EC) No 1924/2006). The antioxidant properties of the insoluble coffee fibre can be associated with phenolic compounds bound to proteins, polysaccharides and melanoidins [49,50]. There is evidence for the existence of a complex dietary fibre-protein-MRP-polyphenols, named "maillardized insoluble dietary fibre" in bakery products [51].

This coffee antioxidant fibre combines beneficial effects of both, dietary fibres and antioxidants. Insoluble fibre may play an important role for weight loss during consumption of a high-fat diet by adhering fat to its complex structure [52,53], and it has been inversely associated with the risk of T2D [54]. The mechanisms of action behind insoluble fibre have been suggested to be more peripheral. An accelerated secretion of GIP hormone, which stimulates postprandial insulin release, is observed directly after the ingestion of insoluble fibre in healthy women. The insoluble fibre can also result in a reduced appetite and food intake [55], thereby decreasing the caloric intake and BMI. The short chain FFAs produced via fermentation of the insoluble fibre, have shown to reduce postprandial glucose responses [56] through the inhibition of glucose metabolism via GLUT 4 transporters [57] and to prevent inflammation [58]. On the other hand, the intake of antioxidant dietary fibre has been recommended for health improvement of the gastrointestinal tract. The pathogenesis of various gastrointestinal diseases such as irritable bowel syndrome and inflammatory bowel disease is partially due to oxidative stress. Antioxidant compounds may be beneficial at reducing the risk of these gastrointestinal diseases [59]. In fact, this is consistent with the current concept that antioxidants act by inhibiting oxidative stress pathways in a tissue- and environment-specific manner and not by simply acting as scavengers [60].

This gluten-free coffee fibre can be used into diverse combinations with other basic and/or novel ingredients such as non-nutritive sweeteners and gluten-free flours. Therefore, it may allow their use for people with special nutritional needs, such as diabetic and overweight or obese people. In this sense, further investigations have been performed and described in the present PhD thesis. New knowledge is provided with regard to the validation of this coffee fibre from SCG as a new food ingredient and its sustainable feasibility to be incorporated in non-added sugar biscuits with potential health-promoting properties for metabolic chronic diseases (***chapter 2, article 2; figure 5; Annex 5***).

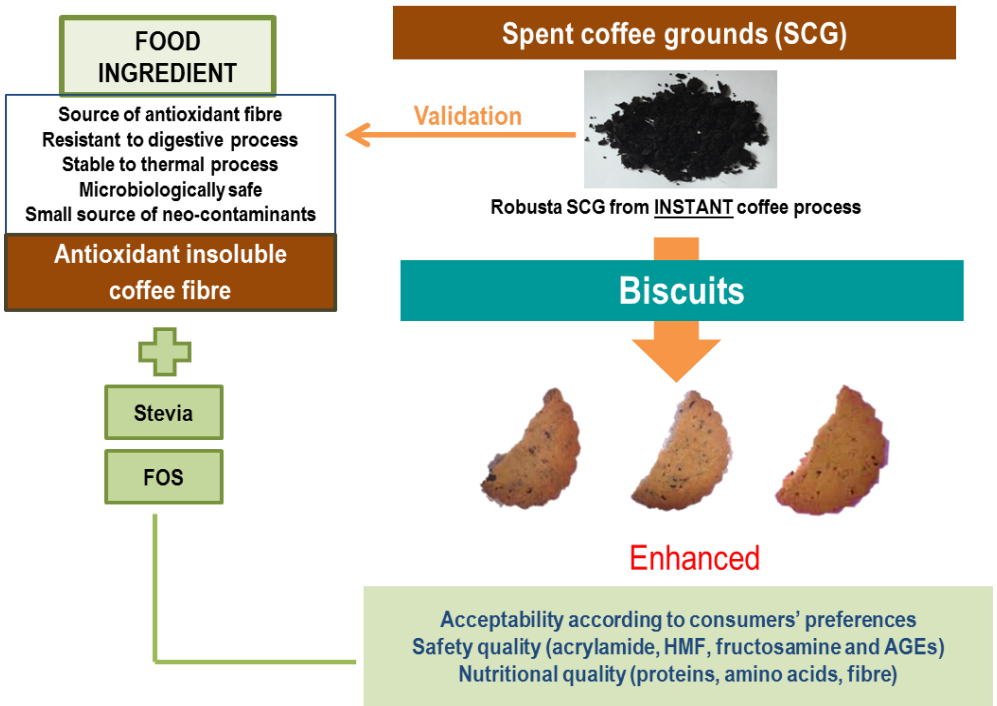

Figure 5. Validation of antioxidant insoluble dietary fibre from SCG as food ingredient and formulation of bakery products.

Our results show that SCG possess a very high amount of insoluble dietary fibre (42%), which is superior to other natural sources of insoluble fibre -black beans (16%), lentils (8%), almonds (12%), whole grains (7%) and some fruits (2-2.6%)-. The coffee fibre is stable to thermal process such as a baking and to the abiotic gastrointestinal digestion *in vitro*. On the other hand, it contains low amounts of free CGA and caffeine, and none levels of free sugar. This coffee fibre is also a small source of neo-contaminants -acrylamide (37 µg/kg) and HMF (61 mg/kg)-. The acrylamide values are 92–96% lower than the indicative values proposed by European Commission (2013) for roast

(200-250 µg/kg) and instant coffee (350-595 µg/kg) [61]. Likewise, HMF levels are also very far from those amounts found in coffee (100–1900 mg/kg) and instant coffee (400–4100 mg/kg) [62]. The coffee fibre is exhausted of these compounds as a consequence of the extraction process during the preparation of soluble coffee beverage. In addition, the coffee fibre derived from the instant coffee production presented high microbiological safety in this particular case. Therefore, SCG become a cost-friendly source of healthy insoluble dietary fibre that does not require further purification to be used as food ingredient of bakery products to enhance their nutritional and biological quality.

Sugar was replaced with non-nutritive sweeteners such as stevia, and FOS were added as soluble dietary fibre and enhancer of the taste of the coffee fibre-containing biscuit (CFB). The sensory and acceptance tests of the novel biscuits show that the coffee fibre and stevia are highly accepted ingredients, and above all when combined with FOS in the formulation. FOS present slightly sweetness and might act masking negative off-flavours from the stevia [63] and coffee fibre [64]. Therefore, these novel biscuits seem to meet consumers' preferences. Furthermore, CFB exhibited lower levels of both, dietary early MRPs and AGEs, than the sucrose-containing biscuit (SCB). This indicates that the presence of sucrose during baking increases the amount of compounds that can be limited by the replacement with non-nutritive sweeteners. In addition, coffee fibre contains CGA and other phenolic compounds (2 mg/g), contributing one CFB (≈10 g) in 0.4 mg CGAs. CGA possesses antiglycative properties (IC50 = 0.4 mg/ml) [36], thus, this coffee fibre might also prevent the formation of MRPs and AGES associated with oxidative stress and inflammation, and eventually causing higher risk of most chronic diseases, such as T2D and obesity [65,66]. Incorporation of FOS to the biscuits also contributes to the balance of soluble: insoluble dietary fibre and promotes the growth of specific beneficial gut bacteria [67]. Recently, relationship between non-digestible carbohydrates, including FOS, and reduction of post-prandial glycaemic responses was established by EFSA (2014) [68].

Additionally, *in vitro* effects of the bioaccessible food components released during the simulated human digestion of the novel CFB, on α-glucosidase activity and satiety hormones were examined. To the best of our knowledge, this is the first report on the potential antidiabetic and satiating effects of foods comprising coffee fibre and non-nutritive sweeteners (***chapter 2, article 3; figure 6***).

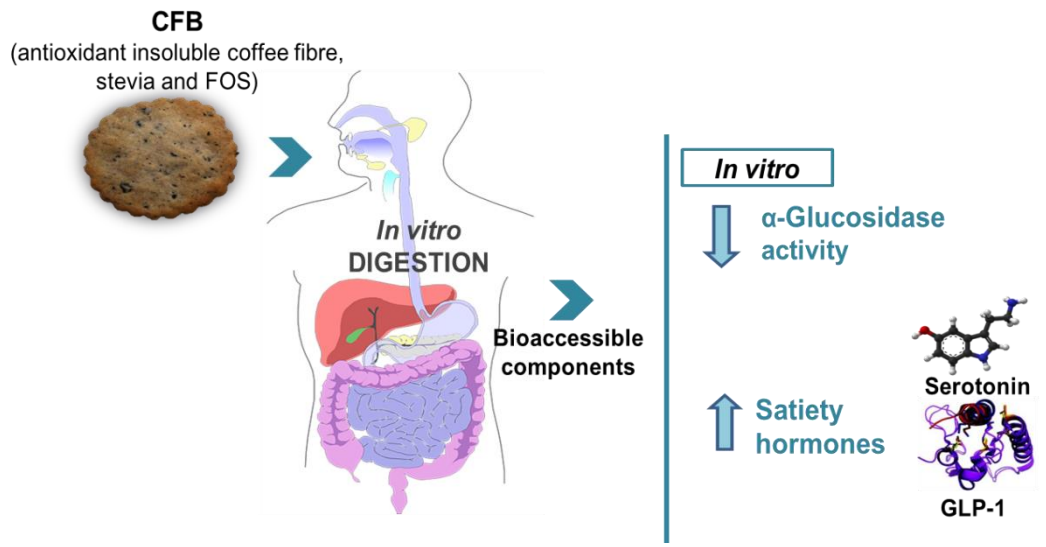

Figure 6. Biological effects of novel coffee fibre-containing biscuit (CFB) on glucose tolerance and satiety.

The content in glycaemic sugars such as glucose and fructose in the biscuit digests was significantly reduced. The CFB can be classified as "no added sugars" declaring that may "contains naturally occurring sugars" (European Regulation (EC) No 1924/2006). Likewise, the content of insoluble and soluble dietary fibre of the novel biscuits provided by the coffee fibre from SCG and FOS respectively, lead to categorize these novel biscuits under the nutrition claim "high fibre" (≥ 6 g of fibre per 100 g or ≥ 3 g of fibre per 100 kcal). Recent studies have concluded that foods high in fibre and whole grains enhance satiety when consumed as snacks [69].

The present study shows antioxidant character of the CFB digest. The transportation of dietary antioxidants through the gastrointestinal tract has been described as an essential function of dietary fibre. Polyphenols linked to dietary fibre may be released in the colon by the action of the microbiota, producing bioactive metabolites and an antioxidant environment, thereby reducing the risk of gastrointestinal diseases associated with oxidative stress and inflammation [70] (***figure 6 (i)***). CGA, which is present in the coffee fibre, has shown potential to inhibit signalling molecules involved in inflammation processes, thereby acting as an anti-inflammatory antioxidant compound [58].

Stevia and FOS present in the novel biscuit were effective inhibitors of α-glucosidase activity *in vitro* (***figure 6 (ii)***). Moreover, previous studies have reported an association of antioxidants, in particular polyphenols, with α-glucosidase inhibition [71]. The release of phenolic compounds incorporated in the coffee fibre structure during the digestion process may enhance tolerance to carbohydrates by inhibiting intestinal α-glucosidase. Diterpens such as cafestol and kahweol, which have been

described to be present in SCG [72], might be contributing to this inhibitory effect on α-glucosidase exerted by the novel biscuits [73]. Alpha-glucosidase inhibitors prevent the degradation of complex carbohydrates into glucose, and carbohydrates remain longer in the intestine, reducing their impact on blood sugar and therefore postprandial hyperglycaemia. The control of glucose absorption plays a key role in the management of T2D.

The coffee fibre exhibited a potent stimulation of the gut serotonin and GLP-1 hormones *ex vivo* (***figure 6 (iii)***). These intestinal hormones can regulate the feeling of fullness via neural paracrine routes with subsequent afferent signalling to brainstem nuclei [74,75]. GLP-1 also regulates the feeling of fullness via the endocrine pathway through the hepatic portal and cava vein [74]. The intestinal secretion of serotonin responds to chemical and mechanical stimuli after food intake [76]. Thus, the antioxidant coffee fibre is expected to exhibit a greater stimulation of satiety hormones *in vivo* than that described in this study, due to the physical effect of the indigestible material obtained from the digestive process. In addition, the soluble fibre (FOS) incorporated to the novel biscuits may be also positively affect the release of these satiety hormones. It has been described, in a double blind randomized clinical trial, an increase of the satiety feeling when FOS enriched cookies (10 per day) were consumed by obese patients for a month [77]. On the other hand, galactomannan, a soluble fibre that is released from the SCG during the digestive process, may enhance satiety by forming a viscous gel in the stomach, and thereby slowing gastric emptying and enhancing fullness [57]. It has been suggested recently the potential of eating high-fibre snacks between meals to promote satiety and suppress overconsumption at the subsequent meal [69].
The secretion of GLP-1 hormone also participates in glycaemic tolerance via glucose-induced secretion of insulin from pancreatic β-cells and via glucagon release inhibition from pancreatic α-cells [78] (***figure 6***). Therefore, a double antidiabetic and satiating potential effect might be achieved thanks to the secretion of GPL-1.

Other ingredients and compounds released during the digestion of the novel biscuit such as gluten peptides might also be involved in the described biological effects. Further research is required in order to find out which other compounds released during the digestion of the novel biscuits, comprising coffee fibre and non-nutritive sweeteners, are responsible of the potential effects on satiety and glucose tolerance. Clinical studies should be also conducted to evaluate their effect *in vivo*.

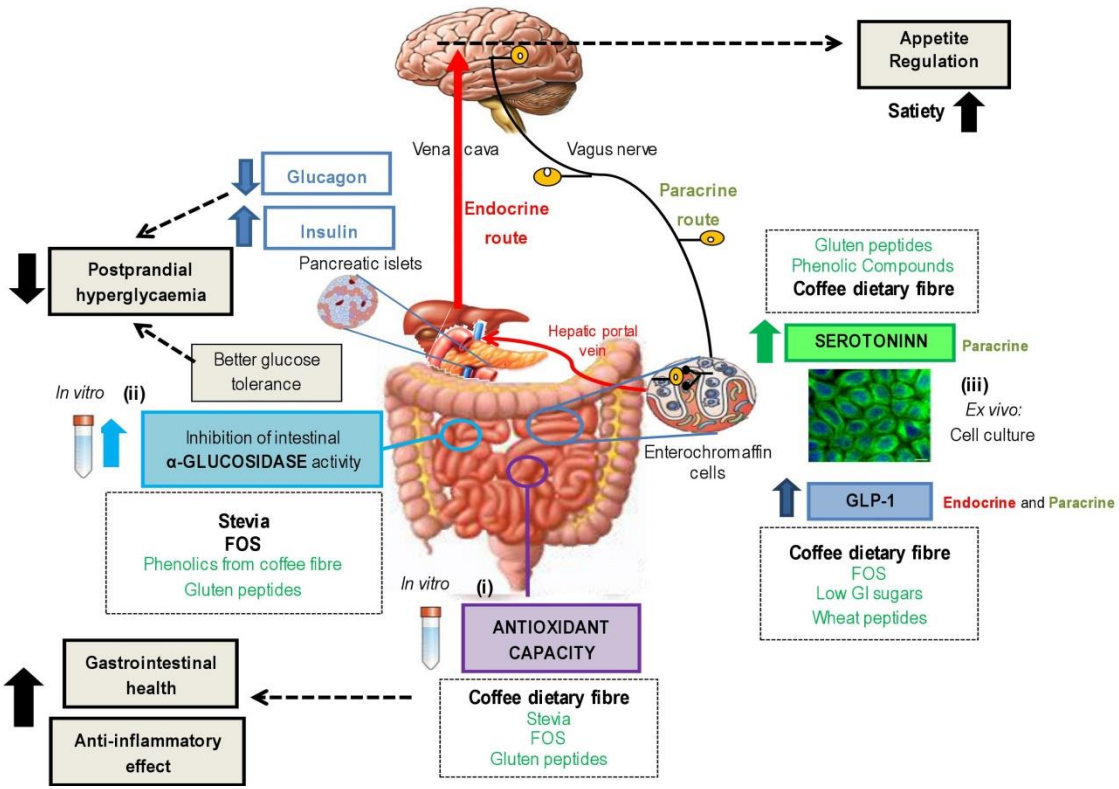

Figure 6. Pathways of action of the novel coffee fibre-containing biscuit (CFB): (i) antioxidant capacity, (ii) inhibition of intestinal α-glucosidase and (iii) secretion of satiety hormones. Ingredients shown to have *in vitro* biological effects in the present study are represented in black, and those with potential physiological effects described in the literature are represented in green.

3. Impact of the digestive process on the release of compounds associated with chronic diseases

MR has been traditionally described to occur in foods during cooking. This non-enzymatic reaction provides compounds responsible of the colour and flavour but also neo-contaminants such as acrylamide and HFM, and other compounds with health impact. The MR occurring in foods generates numerous of heterogeneous compounds, such as Amadori or Heyns products corresponding to the early stages, and AGEs from the advanced steps. All of them are the so-called dietary MRPs. It has been observed that hyperglycaemia state in diabetic people contributes to an accelerated non-enzymatic glycation in the body and thereby, to the endogenously formation of AGEs [66]. Dietary MRPs are partially absorbed by the intestine contributing to those endogenous formed [79,80] thus, increasing the pool of AGEs found in the human body. These compounds derived from the glycation reactions have been associated with oxidative stress and inflammation, processes that eventually cause most chronic diseases, including diabetes [81].

Due to the increased intake of fructose as HFCS and its relationship with different chronic diseases such as asthma, bronchitis and arthritis, it has been hypothesized that these associations might be mediated through the intestinal *in situ* formation of AGEs and their subsequent absorption [82]. Therefore, the gastrointestinal tract would become another spot of MRPs' formation if this hypothesis was confirmed. In this sense, the present research aimed to study the formation of these compounds during *in vitro* oral gastrointestinal digestion [46] of simplified meal systems comprising an average meal and sugar-containing meals (***chapter 3; figure 7***). This is the first report that confirms this event under the studied conditions.

The gastric phase of the digestive process (pH 2, 37º C, 1 h 30 min) is essential to break the dietary proteins and release free amino acids or peptides to the medium. These reactive amino groups react with the carbonyl groups of the sugars present in the intestine, whose conditions (pH 7, 37º C, 2 h 30 min) seem to favour the reaction between them. The reactivity of sugars and amino acids have been largely studied over the past years, however, this study is the first described under human digestive conditions. Moreover, the reaction comprises not only the formation of MRPs from the amino groups of the arginine and lysine side chains, but also the alpha amino groups of all the released amino acids. Fructosamine and fluorescent adducts were detected in digests of those meals containing HCFS and starch, respectively. CML and MGO-derivative AGEs were found in the meals composed of fructose and only MGO-derivative AGEs in presence of glucose. Physiological concentrations (43

mM) of sugars in simplified systems composed by single amino acids caused formation of MRPs under intestinal conditions. Fructose (43 mM) gave rise to CML by interaction with lysine, which was observed within 1 hour of incubation at intestinal conditions. This result is remarkable since emphasizes the MR is taking place very fast, which has not been recognized widely in the past.

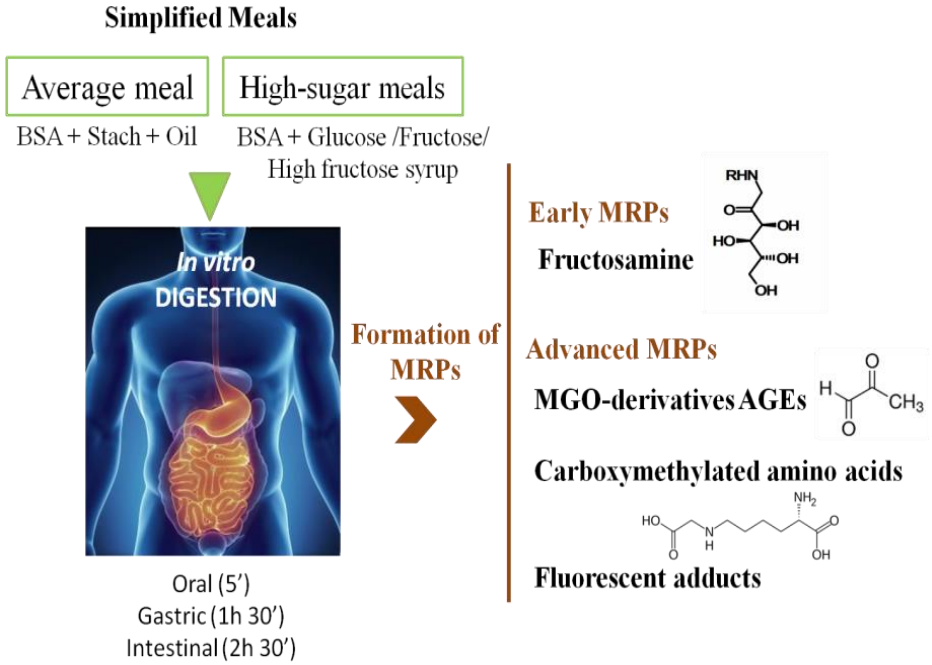

Figure 7. Maillard reaction products (MRPs) generated during gastrointestinal digestion of an average and sugar-containing meals.

There is evidence that more excess of free fructose is being consumed than has been assumed. Results from a study indicate that popular soda beverages are sweetened with a HFCS variant that is 60% fructose and 40% glucose, rather than the 55/45 formula that is generally recognized as safe [83,84]. These findings raise the possibility that daily excess free fructose intakes exceed dosages that have been correlated with fructose malabsorption [85,86]. Notably, equal amounts of fructose and glucose monomers are not associated with adult fructose malabsorption [87]. However, HFCS is not solely responsible of the glycation reaction occurring in the intestine. Our results show that accumulation in the intestinal lumen of a theoretical maximal concentration of glucose (314 mM) as the result of complete digestion of the average meal containing starch promotes the formation of MRPs as well. A delay on the absorption of the sugar due to either fructose malabsorption or change in intestinal membrane functionality because of the presence of fat [88,89] seems to favour these

reactions. Therefore, the intake of sugar-containing meals, in presence of dietary fat or malabsorption state will probably develop the formation of these AGEs-derivatives *in situ*.

Considering the above information, the reformulation of foods plays a key role to lessen the formation of MRPs during the human digestive process. Fully and readily digestible carbohydrates such as glucose and starch promotes a rapid increase in blood glucose or glycaemic response. Foods containing high amount of these available carbohydrates are considered as high GI and GL foods. It is the case of highly processed starchy foods, which will probably impact on the available glucose concentration in the small intestine due to their fast digestibility, thereby favouring the formation of MRPs *in situ*. The intake of low GI and GL foods appears as an effective alternative to reduce this event, since non-digestible carbohydrates are resistant to hydrolysis in the small intestine and therefore, they are not bioaccessible. EFSA has issued a favourably opinion for resistant starch and FOS being considered to have effect on reduction of postprandial glucose response [68]. Resistant starch can be used to replace flour on a 1 for 1 basis without significantly affecting dough handling or rheology because of its fine particle size. Its physical properties provide crispiness and improved texture in the end-product. Resistant starch has been successfully used in a range of baked products with reduced sugar content [90]. Coffee flour (http://www.coffeeflour.com/) obtained from the coffee fruit possess high content of dietary fibre (50%) and low glycaemic sugars that make it excellent to replace normal flour in bakery products as well. It may reduce the release of compounds associated with chronic diseases during the digestive process. Likewise, the use of non-nutritive sweeteners as replacers of sucrose, as well as the sustainable use of coffee by-products such as CS as food ingredient of bakery products might limit the glycation of the amino acids and peptides released during the digestive process [91]. Further studies should be performed to confirm these preliminary findings by employing *in vivo* assays.

4. Future perspectives

Before the legal authorization of the health claims of functional foods or food components, the Panel on Dietetic Products, Nutrition and Allergies (NDA) of the EFSA demands evidence from all the studies performed, including *in vitro*, animal and human studies [92]. Human intervention studies are only feasible once the product is already developed and it is necessary to establish the dose–exposure relationship (pharmacokinetic studies) and at last the exposure–response relationship (pharmacodynamic studies) (***figure 8***).

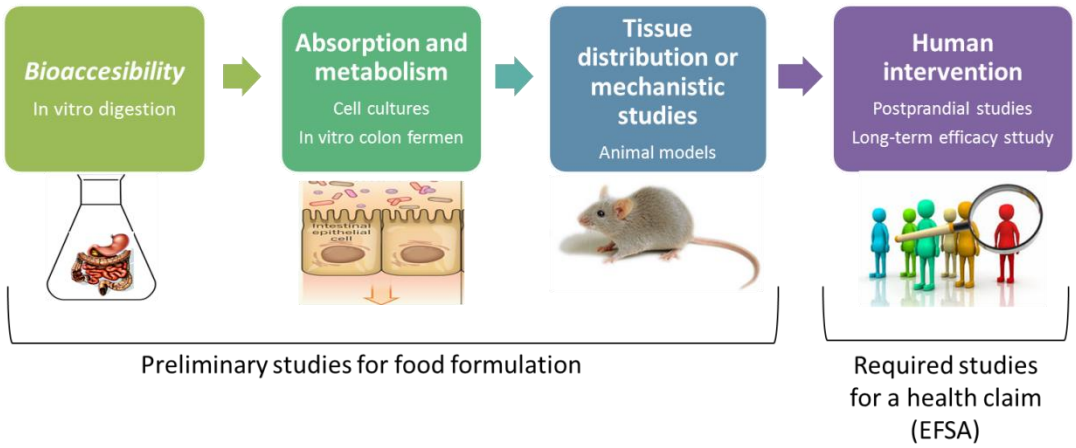

Figure 8. Steps for the authorization of health claims in functional foods: from the *in vitro* assays, recommended in early stages of the development of a functional food, to human intervention studies, which are aimed to establish the dose–exposure relationship and the exposure–response relationship.

In this context, the present PhD thesis provides novel scientific information supporting the usefulness and validation of the coffee by-products, CS and SCG, as food ingredients of novel beverages and bakery foods. Moreover, the biological effects *in vitro, ex vivo* or employing animal models, of these novel foods and ingredients to reduce the risk of obesity and T2D are presented. Likewise, it is demonstrated the importance of the digestive process in the release of bioactive components and/or the formation of compounds associated with metabolic chronic diseases *in vitro*.

"Promotes satiety" or "helps to manage body weight" could be some of the statements of health claims related to the novel beverages and foods presented in this thesis. More research would be necessary to complete the whole process for an authorized health claim. Studies in animals to confirm the satiating and antidiabetic effect are required, as well as postprandial human studies to assess the effects of the dose. Long-term, randomized, controlled, dietary intervention trials with appropriate compliance biomarkers are also necessary in order to assess the full and unequivocal role that the bioactive compounds play in preventing chronic human disease.

Furthermore, future research on the gastrointestinal benefit effects of the patented antioxidant coffee fibre might be of interest. Studies *in vivo* and clinical trials on the potential protection against inflammatory chronic diseases such as irritable bowel syndrome, as well as its mechanisms of action of the coffee fibre may be a remarkable future research line.

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Conclusions

CONCLUSIONS

From the work performed in the present PhD thesis can be deduced the following conclusions:

1. The use of CS for the preparation of novel beverages has been proposed for the first time. It has been obtained an antioxidant beverage with acceptable sensorial properties and reduced sugar content, which meet current consumer's demands and global requests on the sugar content reduction.

2. The novel antioxidant beverage based on CSE reduces body fat accumulation *in vivo*. Physiological active concentrations of caffeine and CGA in the beverage contribute to this effect. RCSE beverage exhibits similar effects to those of a commercial dietary supplement made of Robusta decaffeinated green coffee extract.

3. For the first time, full recovery of CS by means of the green conversion into two different products, natural colouring and source of dietary fibre, has been proposed.

4. The use of CSE together with its solid residue, as food ingredient, improves technological, nutritional and sensorial properties of non-added sugar biscuits. The biscuits based on sustainable coffee ingredients show greatly reduced HMF and non-bioaccessible acrylamide levels.

5. SCG from the industrial instant coffee process were proposed as sustainable source of antioxidant insoluble dietary fibre for human consumption. The coffee fibre is stable to baking and to the abiotic gastrointestinal digestion *in vitro*. It presents none levels of free sugar and low amounts of free CGA, caffeine, acrylamide and HMF. As a consequence, it can be considered as a low cost safe food ingredient.

6. CFBs containing non-nutritive sweeteners and FOS, present similar sensorial acceptance to that observed for commercial biscuits with high consumer adhesion in the market, and possess reduced levels of health-impact compounds such as food processing contaminants and AGEs.

7. CFB digests present higher antioxidant character and significantly reduced glycaemic sugars compared to SCBs, which may be beneficial for human health

8. The bioaccessible food components, released during the simulated human digestion of the novel CFB, inhibit *in vitro* α-glucosidase activity and promote the release of satiating hormones, serotonin and GLP-1, in Caco-2 and HuTu-80 cells, respectively.

9. The formation of early MRPs and AGEs associated to the pathogenesis of diabetes, during *in vitro* abiotic gastrointestinal digestion process of model systems mimicking sugar-containing and average meals, respectively, has been described for the first time.

In summary, the present PhD thesis confirms the feasibility of using the coffee by-products, CS and SCG, in the development of novel beverages and foods with enhanced technological, nutritional and sensorial quality as well as with biological effects to reduce the risk of obesity and T2D. It has been achieved the production of novel sustainable health promoting ingredients and foods. In addition, new knowledge on the impact of the digestive process on the release of health-impact compounds has been generated.

Conclusiones

CONCLUSIONES

Del trabajo realizado en la presente tesis doctoral se deducen las siguientes conclusiones:

1. Por primera vez se propone el uso de cascarilla de café para la elaboración de nuevas bebidas. Se ha obtenido una bebida antioxidante con propiedades sensoriales aceptables, y contenido reducido en azúcar que responden a la actual demanda del consumidor y a la solicitud a nivel mundial de reducir el contenido de azúcar.

2. La nueva bebida antioxidante a base de extracto de cascarilla de café reduce la acumulación de grasa corporal *in vivo*. Concentraciones fisiológicas activas de cafeína y ácido clorogénico en la bebida contribuyen a este efecto. La bebida de extracto de cascarilla Robusta presenta efectos similares a los de un suplemento dietético comercial preparado con extracto de café verde Robusta descafeinado.

3. Se ha propuesto por primera vez la recuperación completa de la cascarilla de café mediante su conversión sostenible en dos productos diferentes, un colorante natural y fuente de fibra dietética.

4. El uso de extracto de cascarilla junto con su residuo sólido, como ingrediente alimentario, mejora las propiedades nutricionales y sensoriales de las galletas sin azúcares añadidos. Las galletas basadas en ingredientes de café sostenibles, presentan una reducción significativa de hidroximetilfurfural y acrilamida no bioaccesible.

5. Se proponen los posos de café procedentes del proceso de café instantáneo como fuente sostenible de fibra dietética antioxidante insoluble. La fibra de café es estable al horneado y a la digestión abiótica gastrointestinal *in vitro*. Ésta presenta niveles no significativos de azúcar y pequeñas cantidades de ácido clorogénico y cafeína libres, acrilamida y hidroximetilfurfural. En consecuencia, se considera la fibra de café como un ingrediente alimentario seguro de bajo coste.

6. Las galletas elaboradas con fibra de café, las cuales contienen edulcorantes no nutritivos y FOS, presentan una aceptación sensorial similar a la observada para galletas comerciales con alta adhesión del consumidor en el mercado, y poseen niveles reducidos de compuestos de impacto para la salud tales como contaminantes del procesado de los alimentos y productos avanzados de la reacción de Maillard.

7. Los digeridos de las galletas de fibra de café presentan mayor carácter antioxidante y azúcar glicémicos significativamente reducidos comparado con las galletas de sacarosa, lo que podría ser beneficioso para la salud.

8. Los compuestos bioaccesibles liberados durante la digestión humana simulada de la nueva galleta de fibra de café, inhiben la actividad α-glucosidasa *in vitro* y promueven la secreción de hormonas de saciedad, serotonina y péptido similar al glucagón tipo 1 (GLP-1), en células Caco-2 y HuTu-80, respectivamente.

9. Se ha descrito por primera vez la formación de productos de la reacción de Maillard, de las etapas iniciales y avanzadas asociados con la patogénesis de la diabetes, durante la digestión abiótica gastrointestinal *in vitro,* de sistemas modelo que simulan comidas con azúcar y una comida promedio, respectivamente.

En resumen, la presente tesis doctoral confirma la viabilidad de utilizar los subproductos del café, cascarilla y posos de café, en el desarrollo de nuevas bebidas y alimentos con mayor calidad tecnológica, nutricional y sensorial, y con efectos biológicos para reducir el riesgo de obesidad y diabetes tipo 2. Se ha logrado la producción de nuevos ingredientes y alimentos que promueven la salud sostenible. Además, se han generado nuevos conocimientos sobre el impacto del proceso digestivo sobre la liberación de compuestos con impacto en la salud.

Annexes

ANNEXES

Annex 1: Patent

del Castillo, M. D.; Martinez-Saez, N.; Ullate, M. Formulación alimentaria que comprende marros de café y sus aplicaciones. "**Food formulation comprising spent coffee grounds and their applications"**. WO2014128320 A1. 2014.

Annex 2: Publication related to the PhD thesis

del Castillo, M. D.; Martinez-Saez, N.; Amigo-Benavent, M.; Silvan, J. M. **Phytochemomics and other omics for permitting health claims made on foods**. Food Res. Int. 2013, 54, 1237–1249.

Annex 3: Publication related to the PhD thesis

Mesías, M.; Navarro, M.; Martínez-Saez, N.; Ullate, M.; del Castillo, M. D.; Morales, F. J. **Antiglycative and carbonyl trapping properties of the water soluble fraction of coffee silverskin**. Food Res. Int. 2014, 62, 1120–1126.

Annex 4: Publication related to the PhD thesis

Kacem, I.; Martinez-Saez, N.; Chaabouni, S.E. & del Castillo, M.D. **Use of almond shell as food ingredient**. Submitted to Eur Food Res Technol on the 28th of April, 2017.

Annex 5: "In the NEWS"

Nutraingredients.com: Leftover coffee grounds: Unlocking 6m tonnes of unused antioxidant dietary fibre

Foodexecutive.com: Coffee by-product may be used a potential ingredient in bakery products

ANNEX 1

(19) OFICINA ESPAÑOLA DE PATENTES Y MARCAS

ESPAÑA

(11) Número de publicación: **2 489 915**

(21) Número de solicitud: 201330238

(51) Int. CI.:

A23L 1/308 (2006.01)

(12) PATENTE DE INVENCIÓN B1

(22) Fecha de presentación:
21.02.2013

(43) Fecha de publicación de la solicitud:
02.09.2014

Fecha de la concesión:
02.09.2015

(45) Fecha de publicación de la concesión:
09.09.2015

(56) Se remite a la solicitud internacional:
PCT/ES2014/070062

Fecha de publicación de la mención al informe de búsqueda internacional:
09.09.2014

(73) Titular/es:
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS (CSIC) (100.0%)
C/ Serrano, nº 117
28006 MADRID (Madrid) ES

(72) Inventor/es:
DEL CASTILLO BILBAO, Maria Dolores;
MARTINEZ SAEZ, Nuria y
ULLATE ARTIZ, Monica

(74) Agente/Representante:
PONS ARIÑO, Ángel

(54) Título: **FORMULACION ALIMENTARIA QUE COMPRENDE MARROS DE CAFÉ Y SUS APLICACIONES**

(57) Resumen:
Nueva formulación alimentaria que comprende una combinación de marros de café como fuente de fibra dietética insoluble antioxidante y fuente de proteínas, junto con otros ingredientes adicionales, que se utiliza en la elaboración de alimentos sólidos saludables de panadería, bollería y confitería, y entre los que se encuentran pan, bollos, galletas, cereales de desayuno y aperitivos, destinados a la población en general y a personas con requerimientos nutricionales particulares.

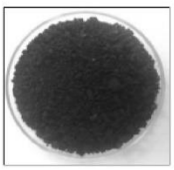

Figura 1

ES 2 489 915 B1

SPANISH NATIONAL RESEARCH COUNCIL (CSIC)

Healthy bakery products with high level of dietary antioxidant fibre

A Spanish public research institution has developed a food formulation based on a coffee by-product that is rich in insoluble antioxidant dietary fibre. This ingredient has application in the food industry, for the manufacture of pastry and confectionery foods as bread, biscuits, and breakfast cereals, among others. Companies interested in the development and commercialization of this food formulation under patent license, are sought.

An offer for Patent Licensing

Valorisation of a by-product of the coffee industry

The coffee industry generates large amounts of by-products rich in carbohydrates, proteins and bioactive compounds which are a low-cost source of these compounds. Moreover, the increase in coffee production makes necessary the search for sustainable strategies of revalorization of these residues.

The developed formulation employs a coffee by-product as a source of antioxidant fibre in a proportion between 4-8 %, in diverse combinations with other basic and/or novel ingredients, as stevia. The resulting formula is rich in insoluble dietary fibre (between 3% and 7%) and its content in acrylamide is low.

Acceptance studies conducted with the cookies made with this formulation have shown more than 70 % acceptance of the panel of tasters for such products.

Main applications and advantages

- Revalorization of a large amount of by-products from the coffee industry, in a sustainable and profitable way.
- Simple and low-cost method: minimum treatment and direct application of the by-product without greatly affecting the original food manufacturing process.
- Application to the development of a healthy product line of pastry, confectionery and/or bakery: bread, buns, cookies, breakfast cereals and snacks, among others.
- The ingredient gives an antioxidant capacity up to 3.3 mg eq. CGA per gram of solid product.
- Use for the elaboration of innovative products for special nutritional needs: its low glycaemic index is ideal for diabetic people and low-energy diets. Its versatility allows the combination with soy flour to make products suitable for celiac people.

Patent Status

Priority established by a Spanish patent application
PCT application filed

For further information please contact

Antonio Jiménez (Ph.D.)
Technology Transfer Manager
Spanish National Research Council
Tel.: (+34) 91 568 19 30
a.jimenez.escrig@csic.es

GOBIERNO DE ESPAÑA MINISTERIO DE CIENCIA E INNOVACIÓN CSIC Alimentos Funcionales Ingenio 2010

ANNEX 2

Food Research International 54 (2013) 1237–1249

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Phytochemomics and other omics for permitting health claims made on foods

María Dolores del Castillo [a,*], Nuria Martinez-Saez [a], Miryam Amigo-Benavent [b], Jose Manuel Silvan [b]

[a] Food Bioscience Group, Department of Food Analysis and Bioactivity, Institute of Food Science Research (CIAL, UAM-CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain
[b] Department of Nutrition and Metabolism, Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), José Antonio Novais 10, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 26 September 2012
Accepted 16 May 2013

Keywords:
Food bioactive components
Foodomics
Health promoting properties
Health claims
Omics approaches
Phytochemicals
Phytochemome
Phytochemomics

ABSTRACT

Various food components positively affect human health and wellness. Phytochemicals have been proposed as health promoters. Several claimed healthy products including foods, dietary supplements, nutraceutics and cosmetics containing phytochemicals are commercialized worldwide. Products based on phytochemicals are nowadays very popular. Phytochemicals' health promoting properties are under evaluation by scientists and regulators' authorities. Phytochemomics is a comprehensive concept aimed to increase the knowledge on phytochemicals' bioactivity and their impact in health, aging and diseases, which is of growing importance in food, medicine and cosmetic sciences. These achievements are based on up-to-date analytical platforms including, but not limited, to mass spectrometric approaches.

Foods are very complex mixtures of bioactive components in different concentrations. Phytochemomics together with other omics are essential for authorizing or rejecting nutrition and health claims made on foods. On the basis of the data collected by using omic approaches a cause-effect relationship may be established between a food category, a food or one of its constituents and the claimed effect.

1. Introduction

According to article 10(1) of Regulation (EC) No. 1924/2006, health claims made on foods are prohibited unless they are authorized by the Commission in accordance with that Regulation and included in a list of permitted claims. One of the objectives of that Regulation is to ensure that health claims are truthful, clear, reliable and useful to the consumer. Permitted health claims must provide scientific evidence on the relationship existing between a food category, a food or one of its constituents and health, the claims should be subject to the same conditions of use indicated for the permitted health claims (Commission Regulation (EU) No. 432/2012).

There is a number of health claims for which either a further evaluation is required before the authority considers their inclusion in the list of permitted claims. The Commission has identified a number of claims submitted for evaluation, referring to effects of plant or herbal substances, commonly known as 'botanical' substances or phytochemicals, for which the Authority has yet to complete a scientific evaluation (Commission Regulation (EU) No. 432/2012). Phytochemicals are very popular as healthy constituents and data on that have to be collected. Phytochemomics may greatly contribute to this knowledge, and its potential for certificating health claims ascribed to phytochemicals is discussed in the present review.

Foods are complex mixtures of bioactive compounds. Each single food component contributes to the overall food bioactivity. The usefulness of phytochemomics and other related omics such as foodomics and lipidomics for a better understating of food components' effects in health is discussed in the present article. Foodomics has demonstrated to be a very useful approach for establishing health claims made on foods (Cifuentes, 2012; Garcia-Canas, Simo, Herrero, Ibáñez, & Cifuentes, 2012; Ibanez et al., 2012; Picariello, Mamone, Addeo, & Ferranti, 2012; Puiggros, Sola, Blade, Salvado, & Arola, 2011; Valdes et al., 2012). The term 'foodomics' has been coined to define studies in the food and nutrition domains through the application of advanced "omics" technologies to improve consumer's well being and health (Cifuentes, 2009). Foodomics is intended to be a global discipline that includes all of the emerging working areas in which food (including nutrition), advanced analytical techniques (mainly omics tools), and bioinformatics are combined.

Food processing, storage conditions and physiological events can dramatically affect food composition and bioactivity. Novel information allowing to understand the effect of all those processes on food and consequently on human health can be obtained by application of omics approaches such as phytochemomics, which is a novel concept proposed

Abbreviations: EFSA, European Food Safety Authority and FDA or USFDA Food and Drug Administration.

* Corresponding author. Tel.: +34 91 0017900x953; fax: +34 91 0017905.
E-mail addresses: mdolores.delcastillo@csic.es (M.D. del Castillo), nur.martinez@titulado.uam.es (N. Martinez-Saez), miryam.amigo@ictan.csic.es (M. Amigo-Benavent), jm.silvan@csic.es (J.M. Silvan).
URL's: http://www.cial.uam-csic.es (M.D. del Castillo), http://www.cial.uam-csic.es (N. Martinez-Saez), http://www.ictan.csic.es (M. Amigo-Benavent), http://www.ictan.csic.es (J.M. Silvan).

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http://dx.doi.org/10.1016/j.foodres.2013.05.014

in the present article. Phytochemomics combines different areas of knowledge.

2. Phytochemomics & health claims made on foods

Fig. 1 shows a schematic representation of those fields covered by the phytochemomics approach. Phytochemomics studies the impact of the phytochemome (Fig. 2) in cell, tissues and biofluids with the final aim to understand its accurate contribution in health, aging and diseases for permitting or rejecting health claims made on phytochemicals. Because phytochemicals are naturally present in tiny concentrations and accompanied by others bioactive compounds their contribution to the overall bioactivity of the food or herbal product is a matter of discussion. Phytochemomics will sort out the doubts on that field. The approach allow to obtain information on phytochemome at different levels (DNA, RNA, proteins, metabolic pathway) by employing advanced analytical approaches such as mass spectrometry (MS) among others.

The phytochemome, as represented in Fig. 2, includes phytochemical forms naturally present in edible plants and medicinal herbs, their modifications determined by processing and those produced by an organism or system by different physiological processes such as digestion, absorption and metabolism. Phytochemicals can be defined, in the strictest sense, as chemicals produced by plants. However, the term is generally used to describe chemicals from plants that may affect health, but are not essential nutrients. There may be as many as 10,000 different phytochemicals having the potential to affect diseases such as cancer, stroke or metabolic syndrome (Scalbert, Manach, Morand, Remesy, & Jimenez, 2005). Phytochemome includes the intracellular and extracellular phytochemical composition; as well as, its structure, activity and mechanism of action.

Several phytochemicals have been approved as health promoters by the EFSA (flavanols, hyroxytirosol, tyrosol, sterol and stanol steres, olive oil polyphenols, soluble and insoluble fiber, resistant starch, guar gum, beta-glucans, pectins, arabinoxylan, glucomannankonjac mannan-), linoleic acid, oleic acid, alpha-linolenic acid) and FDA (sterol and stanol steres, soluble and insoluble fiber, Bowman-Birk inhibitor) (Table 1) and others are under study for achieving the goal (Commission Regulation (EU) No 432/2012). Disciplines such as immunology,

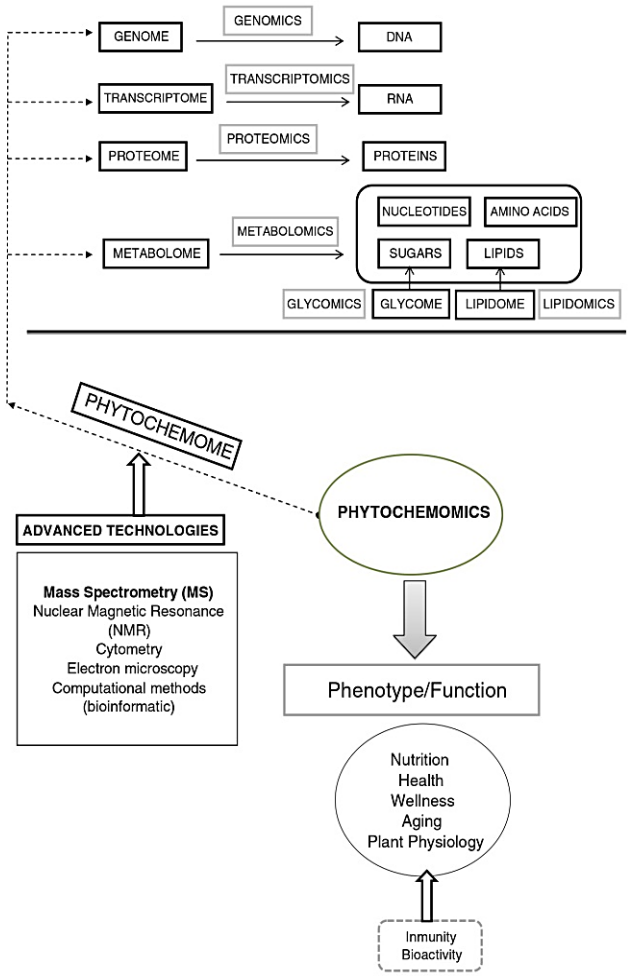

Fig. 1. Overview of phytochemomics platform.

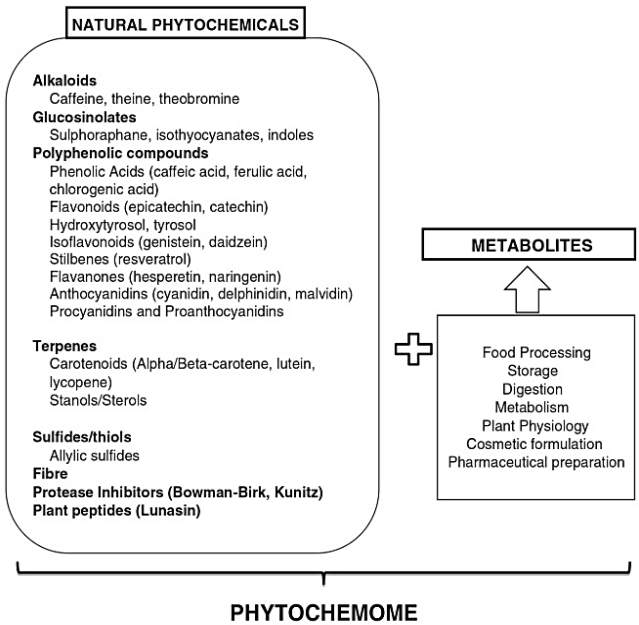

Fig. 2. Schematic representation of phytochemome.

biochemistry and so may be successfully used for acquisition of relevant data for scientific documentation of the effect of phytochemome in health, aging and wellness (Fig. 1).

Phytochemomics can be considered as a separate new discipline for complementing foodomics. Several investigations have been conducted to obtain scientific information for phytochemical health claims. The new discipline has its own identity. Although phytochemicals are part of the daily diet of a great percentage of the population worldwide, with independence of the dietary habits or religious preferences, they can be also treated within the category of traditional drugs or simply bioactive compounds with health promoting properties with applications in other fields beyond food such as medicine and cosmetic. Phytochemicals, for instance, are very relevant in plant physiology and in agriculture. Most phytochemicals are secondary plant metabolites with defense functions. A high number of the health promoting compounds under evaluation or with permitted health claims have a plant origin ('phytochemicals') (Fig. 3). Phytochemomics can be also applied in purification and characterization of phytochemicals from raw plant and agronomical by-products, in development of novel products (food, medicines and cosmetics), in the evaluation of the quality of novel products, functionality, bioactivity and toxicity.

The most commonly analytical platforms for omics are based on MS frequently (Herrero, Simó, García-Cañas, Ibáñez, & Cifuentes, 2012; Picariello et al., 2012) combined with separation methods such as LC, GC and CE. The present article summarizes some examples to demonstrate the utility of MS as advanced analytical approach of omics for collecting accurate data with the final aim to demonstrate phythochemicals health benefits. Phytochemicals with permitted health claims or under evaluation by the authorities to achieve the goal (Table 1) are commented in this manuscript.

2.1. Carotenoids

Carotenoids are pigmented natural compounds synthesized by plants responsible for their color. Fruits and vegetables constitute the major sources of carotenoids in human diet (Fernández-García et al., 2012). Their physiological health activities have been attributed to their antioxidant properties, specifically to the ability to quench singlet oxygen and interact with free radicals (Rao & Rao, 2007). The most investigated dietary carotenoids found in foods in terms of human health are: α- and β-carotenes, lycopene, and lutein (Seren et al., 2008).

Carotenoids are sensitive to light, heat and oxygen for that reason HPLC with UV/Vis or MS detection is the main technique employed for their analysis (Lucini, Pellizzoni, Baffi, & Molinari, 2012; Montesano et al., 2008; Radu, Litescu, Albu, Teodor, & Truica, 2012; Van Breemen, Dong, & Pajkovic, 2012). Recently, a LC–MS/MS method for qualitative and quantitative analysis of lycopene, β-carotene, and α-tocopherol in tomatoes has been proposed (Van Meulebroek, Vanhaecke, De Swaef, Steppe, & De Brabander, 2012). The industrial processing of food rich in carotenoids (juices, sauces, or pastes) involves several treatments that potentially affect the final profile and properties of these compounds and other metabolites in the final commercial product. Phtyochemomics is useful to assess the effect of each separate industrial step. Capanoglu, Beekwilder, Boyacioglu, Hall, and Vos (2008) used both biochemical and metabolomic techniques, LC-QTOF-MS, to assess the effect of each separate step in the industrial production chain starting from fresh fruit to the final tomato paste. On the basis of both antioxidant analyses and broader metabolomic techniques, they detected a gradual and significant decrease in carotenoids upon industrial processing from fruit to tomato paste being most critical events the breaking step, which causes a significant increase in a range of flavonoids and alkaloids, and the pulping step, after which the seed and skin are removed. The investigation performed by these authors is a typical example of the potential of phytochemomics platform for optimization of food processing to achieve healthier foods. Changes in carotenoids concentration in supplemented bread due to processing have been also analyzed by HPLC-MS (Radu et al., 2012).

Beneficial changes can also take place during food processing. For example, carotenoids can exist in *cis* and *trans* isomeric forms, but in tomatoes and tomato-based products, all-*trans* isomeric forms are

Table 1
Phytochemicals proposed as health promoters. *Health claim approved by EFSA. ** Health claim approved by FDA.

Phytochemical	Main examples	Source	Potential benefits
Alkaloids			
	Caffeine, theine, theobromine	Coffe, tea, mate, cocoa	Stimulant to the heart and central nervous system, increase blood pressure Increased fat oxidation leading to a reduction in body fat mass (caffeine) Increased energy expenditure leading to a reduction in body weight (caffeine)
Glucosinolates			
	Sulphoraphane, isothyocyanates, indoles	Broccoli, cauliflower, cabbage, horseradish, Brussels sprouts	Anticarcinogenic and antimicrobial properties Increase activity of detoxifying enzymes Protection of DNA, proteins and lipids from oxidative damage (Sulphoraphane) Hormonal balance and apoptosis of damaged cells (Indole-3-carbinol)
Polyphenolic compounds			
	Flavanols* (epicatechin, catechins)	Green tea, cocoa, grape juices, red wine, apple	Positive effect on human blood flow* (cocoa flavanols) Improvement of endothelium-dependent vasodilation (apple flava-3-ol) antioxidant properties Contribution to the maintenance or achievement of a normal body weight (catechins grean tea) Anti-cancer cell line studies
	Phenolic acids (caffeic acid, ferulic acid, chlorogenic acid)	Apples, pears, citrus fruits, coffee, tea, wine, whole grains, vegetables	Bolster cellular antioxidant defenses Support maintenance of eye and heart health Maintenance of normal blood glucose concentrations(chlorogenic acid) Contribution to the maintenance or achievement of a normal body weight (chlorogenic acid)
	Tyrosols (Hydroxytyrosol, tyrosol)*	Olive oil	Protection of LDL particles from oxidative damage* Maintenance of normal blood HDL cholesterol concentrations Maintenance of normal blood pressure Anti-inflammatory properties Contribute to the upper respiratory tract health Can help to maintain a normal function of gastrointestinal tract Contribute to body defences against external agents
	Isoflavonoids (genistein, daidzein)	Soybeans, soy-based foods, other legumes	Support maintenance of bone and immune health; for women, supports menopausal health
	Stilbenes (resveratrol)	Grapes, red wine, cocoa, chocolate	Anti-cancer cell line studies Protection of DNA, proteins and lipids from oxidative damage
	Flavanones (hesperetin, naringenin)	Citrus fruits	Neutralize free radicals which may damage cells
	Anthocyanidins (cyanidin, delphinidin, malvidin)	Berries, grapes, red wines	Bolster cellular antioxidant defenses; support maintenance of healthy brain function
	Procyanidins and Proanthocyanidins	Cranberry juices, grapes, red wine, tea, cocoa, chocolate, peanuts	Support maintenance of urinary tract health and heart health
	Olive oil polyphenols*	Olive oil	Protection of LDL particles from oxidative damage*
Terpenes			
	Carotenoids (Alpha/Beta-carotene, lutein, lycopene)	Carrots, pumpkin, potatoes, spinach, tomatoes, kale, broccoli, citrus fruits, corn, watermelon	Neutralize free radicals which may damage cells Support maintenance of eye health Anticancer effects, supports maintenance of prostate health
	Stanols/Sterols Esters*,**	Corn, soy, wheat, fortified margarines, green and yellow vegetables, seeds and vegetables oils	Reduce the risk of heart disease. Helps to lower blood total and LDL cholesterol levels** Lowering blood LDL-cholesterol and reduced risk ofheart disease* Maintenance of normal blood cholesterol concentrations*
Sulfides/thiols			
	Allylic sulfides	Garlic, onions, scallions, leeks	May enhance detoxification of undesirable compounds; reduce total and LDL cholesterol
Fibre			
	Soluble**	Oat bran, rolled oats, whole oat flour, oatrim, whole grain barley, dry milled barley, barley betafibre, fruit, vegetables and grain products	May reduce the risk of heart diseases**
	Soluble and Insoluble (both)*,**	Grain products, fruit, and vegetables Barley grain fibre Oat grain fibre Wheat bran fibre Rye fibre Wheat bran fibre	May reduce the risk of some cancers** Increase in faecal bulk* Contributes to normal bowel function* Reduction in intestinal transit time*

Table 1 (*continued*)

Phytochemical	Main examples	Source	Potential benefits
Fibre			
		Arabinoxylan rich-fibre produced from wheat endosperm	Reduction of postprandial glycaemic responses*
	Resistant starch*	All sources	Reduction in the blood glucose rise after meal*
	Guar gum*	Cyamopsis tetragonoloba seeds	Maintenance of normal blood cholesterol levels*
	Beta-glucans*	Oats, oat bran, barley, barley bran, or from mixtures of these sources	Maintenance of normal blood cholesterol levels* Reduction of post-prandial glycaemic responses* (oat and barley)
	Pectins*	Fruits and vegetables	Reduction of post-prandial glycaemic responses* Maintenance of normal blood cholesterol levels*
	Arabinoxylan*	Wheat endosperm	Reduction of post-prandial glycaemic responses*
	Glucomannan (konjac mannan)*	Knojac root plant	Maintenance of normal blood cholesterol levels* Reduction of body weight*
Protease inhibitor			
	Bowman Birk**	Soybean, monocotyledonous and dicotyledonous seeds	**Risk of coronary heart disease and breast cancer, anti-inflammatory, anticarcinogenic
Fatty acids			
	Linoleic acid (ω6)*	Vegetable oils and legumes	Maintenance of normal blood cholesterol levels*
	Oleic acid*	Olive oil	Maintenance of normal blood cholesterol levels*
	Alpha-linolenic acid (ALA ω3)*	Vegetable oils	Maintenance of normal blood cholesterol levels*

predominant. However, during thermal processing, *cis* isomers are formed. Research indicates that more than 50% of the carotenoids found in the human body are present in the *cis* isomeric form, leading to the hypothesis that the *cis* isomeric form is the most bioavailable form in the human body (Boileau, Boileau, & Erdman, 2002). Isomers of carotenoids can be determined employing HPLC-MS/MS in tomato-based processed products such as gazpachos and ketchups (Vallverdú-Queralt, Martínez-Huélamo, Arranz-Martinez, Miralles, & Lamuela-Raventós, 2012). Seven different isomers including trans-lycopene and 5-, 9- and 13-cis-lycopene were identified by these authors.

In biological samples such as human serum and tissues, carotenoids are often present at low concentrations and accompanied by potentially analytical interfering compounds. However, MS strategies have provided an understanding of the absorption carotenoids such as β-carotene through the use of stable isotopes (Fleshman, Riedl, Novotny, Schwartz, & Harrison, 2012). In this case, the authors reported

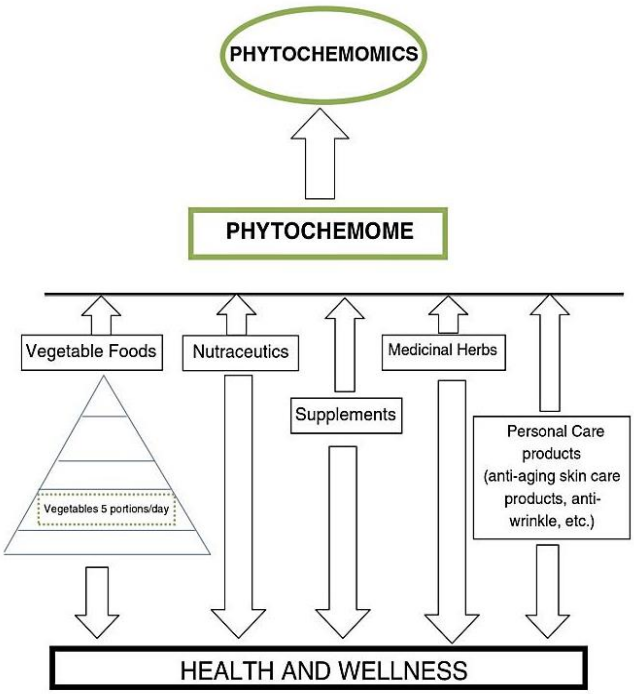

Fig. 3. Diversity of products studied by phytochemomics platform.

an HPLC-MS method that eliminates the complicated sample preparation and allows detecting and quantifying absorbed d8-deuterated-carotene in human plasma after consumption of a single 5 mg dose. The method can be used for relatively high-throughput quantification of the absorption of β-carotene and intestinal conversion of β-carotene to its vitamin A metabolites in humans and may also be useful for the detection of carotenoids in other biological samples. Data on GC-MS using intrinsic labeling have indicated an increase of β-carotene bioavailability in carrot by stir-frying (Ghavami, Coward, & Bluck, 2012).

2.2. Plant sterols

Phytosterols (referred to as plant sterol and stanol esters) are a group of naturally occurring compounds found in plant cell membranes. Stanols are a saturated subgroup of sterols. Whereas about 250 types of phytosterols are actually reported in the literature, nutrition research has focused mostly upon the unsaturated β-sitosterol, campesterol and stigmasterol (Garcia-Llatas & Rodriguez-Estrada, 2011). Fig. 4 shows their characteristic chemical structures. Phytosterols in particular, are important agricultural products used in the health and nutrition industries. They are useful emulsifiers for cosmetic manufacture and supply the majority of steroidal intermediates and precursors for the production of pharmaceutical hormones (Abidi, 2001; Fugh-Berman & Bythrow, 2007). In order to make common food products even "healthier," food manufacturers have taken these phytoesterols from their naturally occurring sources, concentrated them, and added them to common foods that wouldn't normally contain them, such as vegetable oil spreads (margarine), mayonnaise, yogurt smoothies, orange juice, cereals, and snack bars.

Plant sterols and stanols are substances that occur naturally in small amounts in many grains, vegetables, fruits, legumes, nuts, and seeds. Stanols occur in even smaller quantities in many of the same sources. Nuts and vegetable oils can contain more than 1% of phytosterols (Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000). Phytostanols occur in certain cereals (corn, wheat, rye, and rice), fruits and vegetables (Moreau, Whitaker, & Hicks, 2002). Experts have been studying the effects of food fortified with plant sterols for decades. Because phytosterols are structurally similar to the body's cholesterol, when they are consumed they compete with cholesterol for absorption in the digestive system. As a result, cholesterol absorption is blocked, and blood cholesterol levels reduced (Klingberg et al., 2008). Some research works suggest that phytosterols delivered in natural matrices are biologically active at levels present in a healthy diet and have large effects on whole-body cholesterol metabolism (Puiggròs, Solà, Bladé, Salvadó, & Arola, 2011). This health effect has been studied for more than 50 years. The FDA (2010) and EFSA (Table 1) gave these products the status of a "health claim." This means that experts widely agree on the cholesterol-lowering benefits of stanols and sterols. It also allows manufacturers to advertise the heart-healthy benefits on labels. In order to bear the claim, a food should provide at least 0.8 g per day of plant sterols/stanols in one or more servings. Research has not shown any negative health effects of phytosterols. The body's tissues do not retain

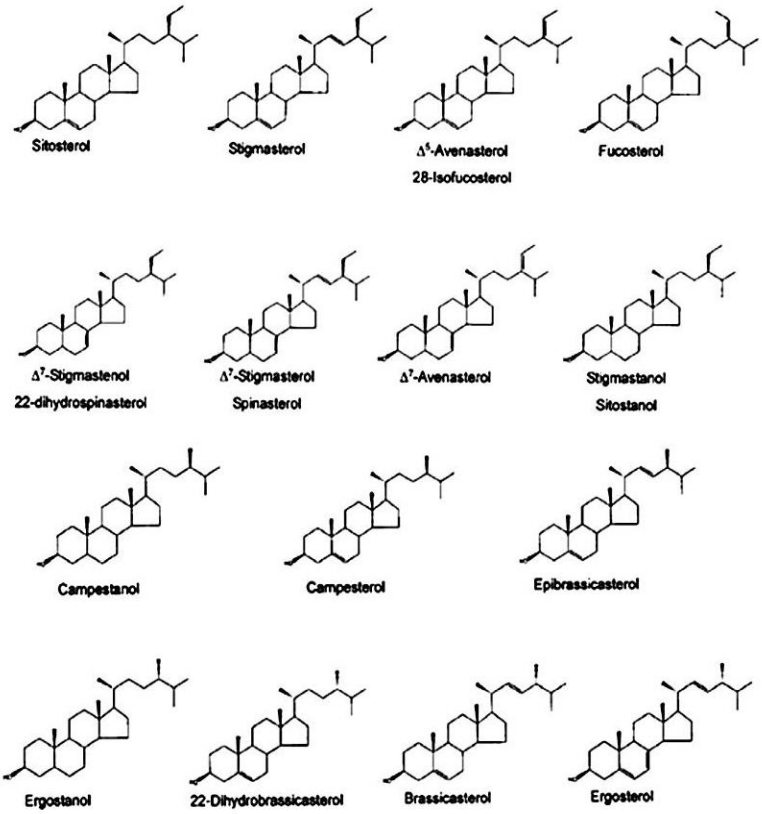

Fig. 4. Chemical structures of phytosterols, phytostanols and their conjugates. The most widely studied phytosterols are stigmasterol, sitosterol and campesterol.

phytosterols, and they do not affect the absorption of fat-soluble vitamins A, D, E and K. There has not been adequate testing to determine the safety of phytosterols in children. Intermittent use is considered GRAS (Generally Recognized as Safe) by the FDA. The ADI (acceptable daily intake) is 130 mg per kilogram (kg) of body weight. Therefore, a child that weighs 50 kg can have up to 6.5 g of phytosterols.

Phytochemomics analytical platforms to assess phytosterols and their metabolites must account for both free and conjugated forms. GC–MS is the best and most widely used tool for the chromatographic separation, identification, and quantification of phytosterols in foods and in biological samples. Phytochemomics platform has been successfully used to determine free phytosterols/stanols as well as of intact phytosteryl/stanyl esters in functional foods such as raw peanuts (Shin, Pegg, Phillips, & Eitenmiller, 2010), cereals (Esche, Barnsteiner, Scholz, & Engel, 2012), or vegetable oils such as, peanut oil (Cherif et al., 2010) and olive oil (Sakouhi et al., 2010). GC–MS allow their correct determination in health promoting milks and yogurts supplemented with either vegetal sterols or stanols (Barnsteiner et al., 2012; Saraiva, Castilho, Martins, Silveira, & Ramos, 2011).

Plant sterols are unsaturated molecules prone to oxidation giving rise sterol oxidation products (POPs). Phytosterols and phytostanols are subjected to oxidation when exposed to air, which can be further enhanced by heating, ionizing radiation, chemical catalysts, fatty acid instauration level, and exposure to light. POPs have been suggested to exert adverse biological effects similar to, although less severe than, their cholesterol counterparts. The metabolism and biological effects of these oxides is also discussed in detail by Ryan, McCarthy, Maguire, and O'Brien (2009) and Garcia-Llatas and Rodriguez-Estrada (2011).

GC-MS has been employed for evaluating stability to oxidation of sterols in commercially available plant sterols-enriched foods after storage and frying process, such as ready-to-eat infant foods (García-Llatas et al., 2008), vegetables oils (Gonzalez-Larena et al., 2011; Orozco, Priego-Capote, & Luque de Castro, 2011) and phytosterols-enriched milk (Menéndez-Carreño, Ansorena, & Astiasaran, 2008). Two-dimensional GC-MS method for the analysis of phytosterol oxidation products in human plasma has been also employed (Menéndez-Carreño, Steenbergen, & Janssen, 2012). The method was successfully validated for the simultaneous quantification of ten POPs in human plasma. The detection limits were below 0.1 ng mL(−1). The concentrations of POPs found in human plasma in that study were between 0.3 and 4.5 ng mL(−1), i.e., 10–100 times lower than the typical values found for cholesterol oxidation products.

LC-MS has been also developed for separating and quantifying phytosterols and steryl fatty acid esters in foods, mainly in vegetable oils. Because sterols are highly lipophilic and have few polar functional groups, they are difficult to ionize by conventional electrospray methods. Atmospheric Pressure Chemical Ionization (APCI) is the most widely used ionization technique for sterol analysis. LC-APCI-MS methodologies have been applied to identify plant sterols in different foods such as olive oil and other vegetables oils (Segura-Carretero et al., 2008; Zarrouk, Carrasco-Pancorbo, Zarrouk, Segura-Carretero, & Fernandez-Gutierrez, 2009). LC-APCI-MS was used to separate and identify 15 sterols and 2 dihydroxy triterpenes in different saponified oils (Segura-Carretero et al., 2008). The authors describes the use of HPLC-APCI-MS to identify efficiently 17 compounds (erythrodiol, uvaol, Δ5, 23-stigmastadienol, 24-methylene cholesterol, brassicasterol, Δ7-avenasterol, cholesterol, Δ5-avenasterol, Δ7-campesterol, clerosterol, campesterol, campestanol, stigmasterol, Δ7-stigmastenol, Δ5,24-stigmastadienol, β−sitosterol and sitostanol) and to quantify seven of them (erythrodiol, uvaol, cholesterol, Δ5-avenasterol, stigmasterol, b-sitosterol and sitostanol) that are legislated upon by several regulations and trademarks laid down by the International Olive Oil Council and the European Union. The methodology was suitable for the identification of 23 compounds belonging to different families (sterols, tocopherols and triterpenic dialcohols) present in olive oil and other kinds of oils, as well as for the quantification of 15 analytes by Zarrouk et al. (2009). Virgin olive oils belonging to six genetic varieties cultivated at La Comunitat Valenciana, Spain (Arbequina, Borriolenca, Canetera, Farga, Picual, and Serrana), were correctly classified with an excellent resolution by using linear discriminant analysis of the HPLC-MS data (Lerma-Garcia, Concha-Herrera, Herrero-Martinez, & Simo-Alfonso, 2009). Ultra-performance liquid chromatography (UPLC)-APCI-MS also provide a fast and reliable strategy for the separation and identification of sterols in foods, such as in vegetable oils (Lerma-Garcia, Simo-Alfonso, Mendez, Lliberia, & Herrero-Martinez, 2010). The optimal conditions were achieved using an Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 μm) with a mobile phase consistent of acetonitrile/water (0.01% acetic acid) using a linear gradient, at a flow rate of 0.8 mL min(−1) and column temperature of 10 °C, giving a total analysis time below 5 min. The determination was performed in selective ion recording mode.

The consumption of products enriched with plant sterols lowers serum total cholesterol concentration, thereby most likely reducing the risk of coronary heart disease. Plant sterols are well known to reduce levels of total cholesterol and LDL cholesterol (De Smet, Mensink, & Plat, 2012). Lipidomics analysis in serum samples from a placebo-controlled, parallel intervention study of 4-week consumption of two plant sterol-enriched yogurt drinks differing in fat content in healthy mildly hypercholesterolemic subjects showed significant effects of the plant sterol intake on the serum lipidome (Szymanska et al., 2012). The study supports that lipidomics, as is suggested in Fig. 1, is very relevant in phytochemomics. Lipidomics has been sucessful applied in the evaluation of food related health effects (Hyötyläinen, Bondia-Pons, & Orešič, in press).

Plant sterol (not plant stanol) enriched products elevates serum plant sterol concentrations in humans. One of the concerns is the potential atherogenicity of elevated serum plant sterol concentrations. The first clue is the presence of coronary atherosclerosis in sitosterolemic patients, characterized by severely elevated serum plant sterol concentrations mostly in the absence of hypercholesterolemia, which suggests that high circulating levels of plant sterols may be atherogenic. The atherogenic effect of plant sterols has to be demonstrated. Phytochemomics may be helpful on that. Fransen et al. (2007) compared serum plant sterols concentrations among users of plant sterol-enriched margarines with those of matched nonusers by GC–MS. Intake of plant sterols was 1.160.6 g/d and was associated with a decrease of serum total cholesterol concentration of 4%, ($P < 0.05$), a change that differed ($P < 0.05$) from the non significant increase in nonusers (2%, $P = 0.16$). Cholesterol-standardized serum sitosterol and campesterol increased in plant sterol users by 22% ($P < 0.0001$) and 103% ($P < 0.0001$), respectively. Cholesterol-standardized serum sitostanol and campestanol increased in plant stanol users by 197% ($P = 0.02$) and 196% ($P = 0.01$). Data were the first to show changes in serum cholesterol, plant sterol, and plant stanol concentrations after (long-term) consumption of plant sterol and stanol enriched margarines in a free-living population in a no experimental setting. Whether the increased serum sterol concentrations result in adverse side effects needs to be investigated in future post-lunch monitoring studies by using phytochemomics.

2.3. Flavonoids

Flavonoids, water-soluble phenolic plant constituents, consist of a wide range of chemicals, including anthocyanins, flavanols, flavones, flavanones, and isoflavones. Phenolic compounds are components of different foods such as fruits, vegetables, cocoa, coffee, tea, and red wine (Table 1). The latest advances in polyphenols and health have been recently reviewed (Tomás-Barberán & András-Lacueva, 2012).

2.3.1. Flavanols

Flavanols are the most structurally complex subclass of flavonoids, ranging from simple monomers, such as catechins and epicatechin, through to complex structures such as proanthocyanidins. They have

been reported to show extensive benefits to human health, including antioxidant, anti-inflammatory, and anti-cancer activities (Crozier, Jaganath, & Clifford, 2009). These phytochemicals are mainly found in green tea, cocoa, grapes, red wines, and berries. Recently, EFSA has approved a health claim for cocoa flavanols, where is concluded that a cause and effect relationship has been established between the consumption of these phytochemicals from cocoa and maintenance of normal endothelium-dependent vasodilation (Commission Regulation N° 432/2012).

LC-ESI-MS/MS is considered the most powerful analytical platform for structural identification of catechins in different foods such as green tea (Bedner & Duewer, 2011; Sultana et al., 2008), cocoa and cocoa products (Calderon, Wright, Hurst, & van Breemen, 2009), rice (Qiu, Liu, & Beta, 2009), grapes and red wines (Nicoletti, Bello, De Rossi, & Corradini, 2008), cranberries (Tarascou et al., 2011), apple pomace (Reis, Rei, & Abu-Ghannam, 2012), spices (Hossain, Rai, Brunton, Martin-Diana, & Barry-Ryan, 2010) and milk-based food products (Redeuil et al., 2009). UPLC–ESI-MS/MS has been used for the identification of catechins, their derivatives and other constituents of foods such as grapes and red wines (Delcambre & Saucier, 2012), or green tea (Guillarme, Casetta, Bicchi, & Veuthey, 2010; Pongsuwan et al., 2008; Scoparo et al., 2012; Spácil, Nováková, & Solic, 2010). Data support that mass spectrometry is a very relevant analytical platform for phytochemomics as it has been previously documented by other omics such as foodomics.

Catechins are extensively transformed during the traditional processing of foods like wines, cocoa or tea. Changes in catechins and other flavonoids during tea manufacture due to oxidation (Chen, Zhang, Lu, & Qu, 2012), fermentation, drying (Kim, Goodner, Park, Choi, & Talcott, 2011; Ku, Kim, Park, Liu, & Lee, 2010; Qin, Li, Tu, Ma, & Zhang, 2012) and packaging (Kim, Welt, & Talcott, 2011) have been successfully followed by LC-ESI-MS. Roasting process of cocoa beans induces epimerization and degradation of catechins. LC–MS allow to characterize the chemical transformation of flavonols brought upon by industrial processing (Kofink, Papagiannopoulos, & Galensa, 2007).

Interactions between catechins and other food components such as proteins, fats, polysaccharides or alcohols, can occur during food processing dramatically affecting their bioavailabity. The effect of different food matrices on the metabolism and excretion of polyphenols is uncertain. The effect of milk on the excretion of (−)-epicatechin metabolites from cocoa powder after its ingestion with and without milk was evaluated by Roura et al. (2007) using LC–MS/MS. One (−)-epicatechin glucuronide and three (−)-epicatechin sulfates were detected in urine excreted after the intake of cocoa beverages. Milk did not significantly affect the total amount of metabolites excreted in urine. However, differences in metabolite excretion profiles were observed; there were changes in the glucuronide and sulfate excretion rates. A very recent study regarding to the effects of chronic cocoa consumption on lipid profile, oxidized low-density lipoprotein (oxLDL) particles and plasma antioxidant vitamin concentrations in high-risk cardiovascular patients suggested that a consumption of cocoa power with milk modulates the lipid profile in high-risk subjects for cardiovascular diseases. In addition, the relationship observed between the urinary excretion of cocoa polyphenol metabolites and plasma HDLc and oxLDL levels suggests a beneficial role for cocoa polyphenols in lipid metabolism (Khan et al., 2012).

Information regarding to absorption and metabolism of catechins in humans after ingestion of different functional foods such as green tea (Del Rio et al., 2010; Sang, Lee, Yang, Buckley, & Yang, 2008; Stalmach, Troufflard, Serafini, & Crozier, 2009; Stalmach et al., 2010; Zimmermann et al., 2009), cocoa and cocoa products (Ritter, Zimmermann, & Galensa, 2010; Urpi-Sarda, Monagas, Khan, Lamuela-Raventos, et al., 2009; Urpi-Sarda, Monagas, Khan, Llorach, et al., 2009), or almond (Bartolomé et al., 2010; Urpi-Sarda, Garrido, et al., 2009) has been obtained by analyzing plasma and urine samples using LC–MS. The action of the colonic microflora is a key part of the overall bioavailability equation and it is an important aspect of bioactivity of dietary flavanols. Roowi et al. (2010) proposed the pathway for the degradation and absorption of green tea flavanols after ingestion of green tea by healthy volunteers. The identification and quantification of these catabolites from flavanols degradation was carried out by GC–MS.

2.3.2. Isoflavones

Isoflavones are considered as phytoestrogens, a group of non-steroid plant constituents that elicit estrogen-like biological response (Dixon, 2004; Murphy & Hendrich, 2002). Isoflavones may reduce risks of cancer, heart disease and osteoporosis and menopausal complications in women (Omoni & Rotimi, 2005). In food based-plants, the most widely studied isoflavones, genistein and daidzein, are usually encountered as conjugates (acetyls, glycosides or malonyls) (Riu et al., 2008) which are hydrolyzed in the human gut to aglycone (biologically active form) (Alves, Almeida, Casal, & Oliveria, 2010). Genistein and daidzein are found mainly in soy and soy-foods. Separation, identification and quantification of isoflavones in foods and their metabolites in human tissues and biological fluids are usually conducted by GC-FID, GC–MS and LC–MS.

Isoflavones in soy functional products (Hong et al., 2011), other beverages (Kuhnle et al., 2008a) and other legumes (Konar, Poyrazoglu, Demir, & Nevzat, 2012) have been identified and quantified by LC–MS/MS. The phytochemomics analysis of isoflavones present in functional foods have focused on only plant-based foods, and there is only little information on foods of animal origin, leading to an underestimation of intake. Kuhnle et al. (2008b) determined the isoflavone content and other phytoestrogens in 115 foods of animal origin and vegetarian substitutes using LC–MS with ^{13}C-labeled internal standards. GC–MS analysis has been optimized for the analysis of isoflavone metabolites in human tissues and biological fluids. However, some researchers have used this technique for the identification of isoflavones in soy-based products such as soymilk (Ferrer, Barber, & Thurman, 2009). Capillary zone electrophoresis coupled with electrospray ionization mass spectrometry (CZE-ESI-MS) has been recently proposed for the separation and quantification of isoflavones in soy products (Bustamante-Rangel, Delgado-Zamarreño, Carabias-Martínez & Domínguez-Álvarez, 2012). The method was applied to the determination of seven isoflavones (glucosides daidzin and genistin, and the aglycones daidzein, genistein, formononetin, biochanin A and glycitein) in soy drink.

Increasing attention has been also paid to the behavior of isoflavones during a variety of food processing technologies. The industrial methods of soybean processing commonly result in significant changes of the isoflavone content in terms of glucoside conjugate concentrations, which may have significant effects on the bioavailability and pharmacokinetics of the isoflavones in human body. Therefore, it is of great interest to investigate the effect of processing technologies (heating, deffating, etc.) on isoflavone profiles during soy-based products production, such as soy flour (Aguiar et al., 2012) and soymilk (Yerramsetty, Mathias, Bunzel, & Ismail, 2011), which has been carried out using LC-ESI-MS/MS.

Due to the impact of isoflavones in human health, the development of appropriate extraction, separation, purification, identification and quantification methods for their determination in biological fluids is extremely important. Data on bioavailability and metabolism of soy isoflavones have been obtained by their analysis in biological samples such as plasma, urine and saliva employing analytical phytochemomics platform. Because isoflavones are specific to soy-based foods and are excreted in urine within 24–36 h of consumption, urinary bioflavonoid assessment is an excellent biomarker for soy absorption. Soy isoflavones metabolites in human urine samples can be undertaken by LC–MS (Morimoto et al., 2011), LC-ESI-MS/MS (Koh & Mitchell, 2011; Yu et al., 2009) and GC–MS (French, Thompson, & Hawker, 2007). Recently, a novel phytochemomics strategy for measuring urinary isoflavones has been developed using LC–MS/MS (Parker, Rybak, & Pfeiffer, 2012). Isoflavones´ biomarkers and the

major metabolites of daidzein and genistein in human plasma have been detected by LC–MS (Setchell et al., 2011) and LC-ESI-MS/MS (Hosoda, Furuta, Yokokawa, & Ishii, 2010). Levels of daidzein, genistein and aquol in urine, saliva and blood of infants after consumption of soy formula were determined by LC–MS/MS (Cao et al., 2009). Data support the utility of MS plataform in phytochemomics.

2.4. Dietary fibre

Nutritionally carbohydrates are classified as digestible, when they can be digested by the hosts' enzymes and absorbed in the small intestine, and non digestible, which cannot be degraded by host enzymes but potentially can be degraded by microbial fermentation (Bach, Skou, & Nygaard, 2012). Permitted health claims made on non-digestible carbohydrates are shown in Table 1.

Dietary fibre is defined by EFSA (2010) as non-digestible carbohydrates plus lignin that comprises: cellulose, hemicelluloses, pectins, hydrocolloids (gums, mucilages, beta-glucans); resistant oligosaccharides (frutooligosaccharides (FOS), galactosaccharides (GOS) and other oligosaccharides that resist digestion); resistant starch and lignin naturally associated with dietary fiber polysaccharides.

Some non-digestible carbohydrates can improve host health by stimulating the growth and/or the activity of beneficial microbiota in the colon and are therefore considered as prebiotics. Other activity ascribed to oligosaccharides is protection versus pathogenic bacteria since they can act as decoys and inhibit pathogens from infecting the host (Barile, Guinard, Meyrand, & German, 2011). Although several health claims have been proposed for carbohydrates, but for now, the EFSA has delivered only favorable verdict for some applications.

FOS are naturally present in foods such as wheat, rye, honey, onion, garlic and banana (Sangeetha, Ramesch, & Prapulla, 2005) and are commercially produced by controlled hydrolysis of inulin. Inulin and FOS are added to foods like infant formulas, fermented milk and dairy products with the hope that they may provide prebiotic benefits (Barile et al., 2011). Products containing these compounds are become very popular and very well accepted by the consumers. However, health claims made on FOS have not been permitted by the authorities.

The feasibility of the omics techniques for identification and analysis of FOS have been documented (Agopian, Aparecida Soares, Purgatto, Cordenunsi, & Lajolo, 2008; Harrison, Fraser, Lane, Villas-Boas, & Rasmussen, 2009; Haska, Nyman, & Andersson, 2008; Mabel, Sangeetha, Platel, Srinivasan, & Prapulla, 2008; Seiper et al., 2008; Sun et al., 2011). Little information regarding to prebiotic FOS resistance to food processing and in vivo physiological events has been published. Some previous studies employing food simplified model have suggested their interactions with other food components during those processes (Amigo-Benavent, del Castillo, & Fogliano, 2010; Golon & Kuhnert, 2012; Mesa, Silvan, Olza, Gil, & del Castillo, 2008).

Whole grain cereals, pulses, fruits, vegetables and potatoes are the main sources of dietary fiber. The list of permitted health claims includes some associated to particular dietary fibre such as guar gum, beta-glucans, pectins, arabinoxylans and glucomannans (Table 1). The chemical structure of some of them can be observed in Fig. 5.

Various analytical platforms may be used to identify the molecular and structural characteristics of polysaccharides in tropical fruits (lychee, mango, banana, pineapple and papaya fruits) such as nuclear magnetic resonance (NMR), ESI-MS, Fourier transform infrared spectroscopy (FTIR), and differential scanning calorimetry (DSC). Starch, cellulose and phenolic compounds with glycosidic structure may be analyzed employing those analytical approaches (Sun et al., 2011). Analysis of oligosaccharides may be performed by MALDI-TOF-MS, ESI-MS, and GC–MS. MALDI-TOF-MS has been employed for identification of molecular ions representing sodiated hexose and pentose oligo/polysaccharides while the analyses by ESI-MS of the signals corresponding to arabinoxylan oligosaccharides with four and five monosaccharide residues showed the presence of isomeric structures differing in degree of branching and localization of the branched residue along the Xylp backbone (Matamoros et al., 2007).

Structure and bioactivity of complex dietary carbohydrates can change due to processing. Polyphenols may form complexes by hidrogen bonding with polysaccharides, arabinoxylans and arabinogalactans and pectin, or proteins such as gluten (Sabanis, Lebesi, & Tzia, 2009). These interactions between both phytochemicals can be investigated by means of MS. Changes in polyphenols occurring in bread supplemented with apple pectin during bread making process were analyzed by LC–MS (Sun-Waterhouse et al., 2011). GC–MS may be also employed to look at the effect of food thermal processing conditions in food complex carbohydrate fraction (Ratnayake, Sims, Newman, & Melton, 2011).

Results published by Eelderink et al. (2012) support the usefulness of omic approaches for evaluation of health claims made on complex carbohydrates. A human study to examine how the *in vivo* starch digestibility is reflected by the glycemic response was conducted by these authors. This measurement is often used as a biomarker to predict starch digestibility. Different meals (pasta with normal wheat bran and bread with normal or purple wheat bran) were enriched in 13C and the dual isotope technique was applied to calculate the rate of appearance of exogenous glucose. The 13C enrichment of glucose in plasma was measured with GC/combustion/isotope ratio MS (IRMS) and LC-IRMS showing similar results.

Recently, an inverse relation between systolic blood pressure and serum 16α-hydroxyestrone, a metabolite of 17β-estradiol, in postmenopausal women, has been found. Formation of 16α-hydroxyestrone is catalyzed primarily by CYP1A2, a cytochrome P450 enzyme. Fruits, vegetables, and grains, which contain more soluble fiber, a known inducer of CYP1A2, as portion of total fiber, was more positively associated with serum 16a-hydroxyestrone legume, which contain less soluble fiber as a portion of total fiber (Patel, Hawkley, Cacioppo, & Masi, 2011). Results seem to indicate that 16α-hydroxyestrone may be a biomarker of the consumption of soluble fiber. The use of enterolignans (phytostrogenic compounds derived from the conversion of dietary lignans by the intestinal microflora) as biomarkers of dietary lignin has been proposed. The study demonstrated a correlation between total lignan intake and plasma enterolignans depending on the type of dietary lignans (Milder et al., 2007). Both studies support the usefulness of phythochemomics for proving the effect of soluble fiber in the human health and the selection of rigth biomarkers for assessing their effects in vivo.

ARs (alkylresorcinol (1,3-dihydroxy-5-alkylbenzene)) are a group of phenolic lipids abundant in the outer fiber layers of rye and wheat grains and are absent in highly refined white flour. It has been proposed that they could function as biomarkers of human whole grain intake (Aubertin-Leheudre, Koskela, Marjamaa, & Adlercreutz, 2008). Remarkably, a significant correlation between plasma ARs and urinary AR metabolites has been shown, and it has been confirmed that urinary AR metabolites are derived from plasma ARs, as has been suggested by other reports (Ross, Becker, Chen, Kamal-Eldin, & Aman, 2005). These data indicate that urinary AR metabolites may be used as biomarkers in epidemiological studies on cereal fiber intake and disease in free-living populations (Aubertin-Leheudre et al., 2008). The example constitutes another evidence of the utility of phytochemomics for the search of new suitable biomarkers of phytochemicals' health effects.

Traditional dietary assessment methods, such as 24-h recalls, weighted food diaries and food frequency questionnaires (FFQs) are highly subjective and impair the assessment of successfully accomplished dietary interventions. Foodomic technologies offer promising methodologies for gathering scientific evidence from clinical trials with sensitive methods to detect and quantify markers of nutrient exposure or subtle changes in dietary patterns (Puiggròs et al., 2011). Similar evidences can be obtained by phytochemomics.

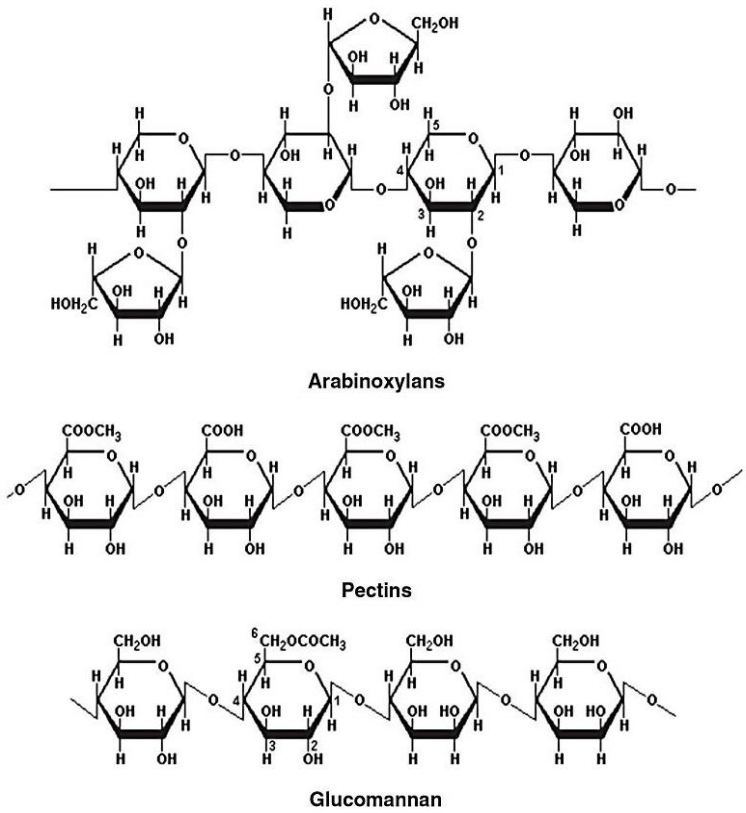

Fig. 5. Illustrative examples of dietary fibres with permitted health claims.

2.5. Proteins and peptides

FDA considers Bowman-Birk inhibitor (BBI) as a drug from 1992. BBI is a small water-soluble protein present in soybean and almost all monocotyledonous and dicotyledonous seeds. BBI is a non-nutrient protein. Several studies have demonstrated the efficacy of BBI against tumor cells *in vitro*, animal models, and human phase IIa clinical trials (Losso, 2008). Phytochemomics may contributes to obtain key information for a wider employment of this non-nutrient protein as a healthier ingredient for formulation of very different products with permitted health claims by European authorities. In fact, Amigo-Benavent, Nitride, Bravo, Ferranti, and del Castillo (2013) have demonstrated that pasteurized orange juice may be an adequate food for application of BBI as functional food ingredient by applying phytochemomics.

In 1999, FDA allowed a health claim on food labels stating that a daily diet containing 25 g of soy protein, also low in saturated fat and cholesterol, may reduce the risk of heart disease (Losso, 2008). Many authors associate this health claim made on soy proteins to the presence of isoflavones bond to the protein structure. Phytochemomics has potential to provide a final answer on that matter and to facilitate the permission for this health claim made on soy proteins by European authorities.

3. Conclusions

Phytochemicals in plant material have raised interest among scientists, producers, and consumers for their roles in the maintenance of human health and in assessing the protective status of people from chronic degenerative disorders. The provision of phytochemical information of a range of foods is vital to support the future work in assessing the protective status of people from chronic degenerative disorders. The authorities have identified a number of claims submitted for evaluation, referring to effects of plant or herbal substances, commonly known as 'botanical' substances or phytochemicals, for which they have not yet to complete a scientific evaluation. Phytochemomics will greatly contribute to complete the job.

4. Future perspectives

At the moment, the list of permitted health claims made on foods included several phytochemicals possessing different chemical structure and bioactivity. This category of compounds may dominate the list of permitted health claims made on foods. As a consequence, the number of commercial available products based on phytochemicals may greatly increase in the future years. More studies to support that ingestion of phytochemicals may be beneficial and safe to human have to be undertaken. Plant sterols, for instance, as ingredients to functional foods are recommended for lowering LDL cholesterol. However, there is an ongoing discussion whether the use of plant sterols is safe that can be concluded by using phytochemomics. The collection of enough scientific data for certificating health claims made on foods is time consuming. The approval of health claims made on phytosterols took decades of scientific work. Lipidomics contribution to this knowledge was fundamental for

permitting this particular health claim made on phytosterols. Time for completing scientific data on health claims made on phytochemicals will be dramatically shortened by phytochemics application and other related omics.

Acknowledgments

Authors thank NATURAGE (AGL2010-17779), A2/036996/11 and FUN-C-FOOD projects for the financial support of their research in Phytochemomics. M. A-B. and J. M. S. thank CSIC and European Social Funds for funding their JAE-Doc contracts. Nuria Martinez-Saez thanks UAM for supporting her research training.

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ANNEX 3

Food Research International 62 (2014) 1120–1126

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Antiglycative and carbonyl trapping properties of the water soluble fraction of coffee silverskin

M. Mesías [a,*], M. Navarro [a], N. Martínez-Saez [b], M. Ullate [b], M.D. del Castillo [b], F.J. Morales [a]

[a] Institute of Food Science, Technology and Nutrition, ICTAN-CSIC, 28040Madrid, Spain
[b] Institute of Food Science Research (CIAL-CSIC-UAM), 28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 6 March 2014
Accepted 30 May 2014
Available online 8 June 2014

Keywords:
Glycation
In vitro glycation inhibitors
Carbonyl trapping
Advanced glycation endproducts (AGEs)
Coffee silverskin extract

ABSTRACT

Carbonyl stress and accumulation of advanced glycation end-products (AGEs) in human tissues are involved in diabetic complications, atherosclerosis, Alzheimer's disease and aging. The objective of this study was to evaluate the *in vitro* protective effect of aqueous extracts of coffee silverskin (CS) in the formation of AGEs and trapping of carbonyl reactive species such as methylglyoxal (MGO). Aqueous extracts of CS from Arabica and Robusta coffee varieties were obtained under environment friendly extraction conditions. CS extracts were characterized by the analysis of dietary fiber, caffeine, chlorogenic acids (CGAs), total phenolic compounds, browning, melanoidins, and antioxidant capacity. CS extracts and CGA exhibited a dose-dependent anti-AGE capacity in the protein–glucose model system (37 °C/21 days) with an IC_{50} of 0.6 mg/mL and 0.4 mg/mL, respectively. Caffeine did not prevent AGE formation under the studied conditions. Regardless to protein–MGO assay (37 °C/14 days), the anti-AGE capacity of CS extracts and CGA was also dose-dependent with an IC_{50} of 1.3 mg/mL and 0.1 mg/mL, respectively. Caffeine weakly inhibited the reaction of protein and MGO. The MGO trapping capacity was established as a model for protection against carbonyl stress. Robusta CS was very effective for the direct trapping of MGO with an IC_{50} of 0.055 mg/mL as compared with Arabica CS (IC_{50} of 0.6 mg/mL). CGA and caffeine showed an IC_{50} for MGO trapping capacity of 0.14 mg/mL and >10 mg/mL, respectively. The highest CGA content in the Robusta CS extract could explain its higher MGO trapping activity as compared with the Arabica CS extract. The anti-AGE and MGO trapping capacities of CS may be associated to other chemical components besides CGA. In conclusion, aqueous CS extract may be considered as a natural source of inhibitors of *in vitro* formation of AGEs and carbonyl stress. The inhibitory effect of the coffee extracts may be associated to their carbonyl trapping capacity.

1. Introduction

AGEs (advanced glycation end-products) are the final products derived from the Maillard reaction or non-enzymatic glycation process produced in the human body. It is known that AGEs are involved in the development of several health disorders such as diabetes and its complications (Vlassara & Palace, 2002), atherosclerosis (Vlassara, 1996), Alzheimer's disease and normal aging (Münch, Thome, Foley, Schinzel, & Riederer, 1997). In addition, the increase in reactive carbonyls in tissues is known as carbonyl stress which leads to directly increase chemical modification of proteins (glycation) and lipids (lipoxidation) in diabetes. Reactive carbonyl species generated from carbohydrate, lipid and amino acid metabolism such as methylglyoxal (MGO), glyoxal, glyoxalaldehyde, dehydroascorbate, 3-deoxyglucosone and malondialdehyde, are potent precursors of AGE formation and protein cross-linking (Thornalley, Langborg, & Minhas, 1999). MGO derived AGE structures, including CEL (N-epsilon-(carboxyethyl)-lysine) and MOLD (methylglyoxal-lysine dimer), are increased in diabetes (Baynes & Thorpe, 1999). Thus, preventing AGE formation/accumulation may control significantly the pathogenesis of diabetes complications.

The inhibition of AGE formation might follow several mechanisms involving, *e.g.*, aldose reductase, antioxidant activity, reactive dicarbonyl trapping, sugar autoxidation inhibition and amino group binding, where the antiglycative activity of phytochemicals has been usually linked to oxidative reactions (Bousová et al., 2005). The inhibition of AGE formation by some synthetic compounds such as aminoguanidine (AG) has been well documented. However, this compound has been associated with several adverse effects in *in vivo* studies (Thornalley, 2003; Williams, 2004) since it is a highly reactive nucleophilic reagent that reacts with many biological molecules (pyridoxal phosphate, pyruvate, glucose, malondialdehyde, and others). Hence, the search for natural products which can inhibit AGE formation has recently been an objective of worldwide research (Peng, Cheng, et al., 2008; Povichit, Phrutivorapongkul, Suttajit, Chaiyasut, & Leelapornpisid, 2010; Wang, Sun, Cao, & Tian, 2009).

Coffee consumption has been associated with reduction in chronic disease risk such as type 2 diabetes (Van Dam & Hu, 2005). Coffee has

* Corresponding author at: Institute of Food Science, Technology and Nutrition, ICTAN-CSIC, José Antonio Novais 10, 28040 Madrid, Spain. Tel.: +34 91 549 2300; fax: +34 91 549 3627.
E-mail address: mmesias@ictan.csic.es (M. Mesías).

http://dx.doi.org/10.1016/j.foodres.2014.05.058
0963-9969/

been suggested as a potential natural source of inhibitors of AGEs. Verzelloni, Tagliazucchi, Rio, Calani, and Conte (2011) stated that coffee melanoidins inhibit the formation of AGEs by (i) acting as a radical scavenger and an Fe-chelator in the post-Amadori phase of the glycation reaction and (ii) inhibiting dicarbonyl reactive compound formation during glucose autoxidation. However, only chlorogenic acid (CGA) effectively inhibits protein glycation and dicarbonyl compound formation.

CS is the tegument of green coffee beans (outer layer) and is the major by-product of the roasting procedure (Napolitano, Fogliano, Tafuri, & Ritieni, 2007). CS is characterized by the presence of high amounts of dietary fiber and antioxidant activity (Borrelli, Esposito, Napolitano, Ritieni, & Fogliano, 2004; Napolitano et al., 2007), and contains several bioactive compounds with potential application in food and health (Pourfarzad, Mahdavian-Mehr, & Sedaghat, 2013). Indeed, CS has been proposed as a functional ingredient due to its health-promoting properties (Esquivel & Jiménez, 2012). The use of aqueous extracts of coffee silverskin (CS) as health promoter has been recently proposed (del Castillo et al., 2013; Martinez-Saez et al., 2014). Among other bioactive compounds, the extract contains CGA and its health benefits have been in part associated to the presence of this bioactive phytochemical. No studies on the effect of the aqueous CS extract as a natural source of scavengers of dicarbonyls and its anti-AGE properties have been reported which are of great interest and it is the goal of the present investigation.

2. Materials and methods

2.1. Materials and reagents

Coffee silverskin from Arabica (*Coffea arabica*) and Robusta (*Coffea canephora*) varieties were provided by Fortaleza S.A. (Spain). According to the manufacturer the weight portion of the coffee silverskin represents 0.5% of the green beans and 0.6% of the roasted beans.

Bovine serum albumin (BSA), 40% methylglyoxal solution (MGO), sodium azide, aminoguanidine (AG), 5-methylquinoxaline (5-MQ), o-phenyldiamine (OPD), 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein disodium, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, ammonium bicarbonate, caffeine (CFF) and chlorogenic acid (CGA) standards were purchased from Sigma (St. Louis, MO, USA). Folin–Ciocalteu reagent, iron(III) chloride, sodium phosphate monobasic, sodium bicarbonate, hydrogen peroxide, sodium chloride, chloroform, and hydrochloric acid were obtained from Panreac (Madrid, Spain). 2,2′-Azobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tri(2-pyridyl)-striazine (TPTZ), 2,2-azobis (methylpropionamidine) dihidro (AAPH) and piridoxamine were purchased from Fluka Chemical (Madrid, Spain). Fiber kit was from Megazyme International Ireland Ltd. Methanol, acetonitrile, glacial acetic acid, potassium hexacyanoferrate and zinc sulfate were purchased from Merck (Darmstadt, Germany). Milli-Q water used was produced using an Elix3 Millipore water purification system coupled to a Milli-Q module (model Advantage10) (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

2.2. Equipments

Synergy™ HT-multimode microplate reader with an automatic reagent dispense and temperature control from Biotek Instruments (VT, USA), capillary electrophoresis apparatus Agilent G16000A (Agilent, Madrid, Spain) and HPLC Shimadzu (Kyoto, Japan) equipped with a LC-20AD pump, a SIL-10ADvp autosampler, a CTO-10ASVP oven, and a DAD (SPD-M20A).

2.3. Preparation of soluble extracts from coffee silverskin

Arabica and Robusta CS extracts were prepared by aqueous extraction according to the procedure patented by del Castillo et al. (2013b). Briefly, 50 mL of boiling water was added to 2.5 g of coffee silverskin. The mixture was stirred at 250 rpm for 10 min, filtered by Whatman paper no. 4 and the filtrate was freeze dried. The powdered extracts were stored in dark and dry place until analysis.

2.4. Determination of total, soluble and insoluble fiber

Total, soluble and insoluble dietary fibers of CS extracts were determined by an enzymatic–gravimetric method based on the AOAC methods 991.43 and 985.29 (Lee, Prosky, & De Vries, 1992; Prosky, Asp, Schweizer, Devries, & Furda, 1988, 1992) and employing a commercial kit. All measurements were performed in triplicate. Results were expressed as mg fiber/g CS extracts.

2.5. Determination of melanoidins

Melanoidin content of CS extracts (1 mg/mL) was determined according to Silván, Morales, and Saura-Calixto (2010). Extracts were then subjected to ultrafiltration using a Microcon YM-10 regenerated cellulose 10 kDa (Millipore, Bedford, MA) at 12000 *g* for 10 min. Melanoidin content was measured spectrophotometrically at 405 nm. Results were expressed as mg/g CS extracts.

2.6. Determination of CGA and caffeine

The procedure for the determination of CGA and caffeine was performed according to del Castillo, Ames, and Gordon (2002). The separation was carried out in a capillary electrophoresis system provided with an ultraviolet visible detection system. Calibration curves of caffeine and CGA were constructed. The analyses were performed in triplicate. Results were expressed as mg/g CS extracts.

2.7. Browning determination

Browning was measured at 405 nm using a microplate reader. CS extracts were dissolved in water at 10 mg/mL in order to obtain an absorbance reading less than 1.5 arbitrary units. All the measurements were made in triplicate. Results were expressed as absorbance units/g CS extracts.

2.8. Determination of total phenolic content

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method as described by Contini, Baccelloni, Massantini, and Anelli (2008). CGA calibration curve was used for quantification. All measurements were performed in triplicate. Results were expressed as mg CGA equivalent/g CS extract.

2.9. ABTS +. assay

ABTS +. assay estimated in terms of radical scavenging activity was employed for determining the antioxidant capacity of the extracts as described by Mesías, Navarro, Gökmen, and Morales (2013). Absorbance reading was taken using a microplate reader. Aqueous solutions of Trolox at various concentrations were used for calibration. All measurements were performed in triplicate. Results were expressed as μmol Trolox equivalent antioxidant capacity (TEAC)/g CS extract.

2.10. ORAC assay

ROO• scavenging activity was measured by monitoring the fluorescence decay as result of ROO-induced oxidation of fluorescein, known

as the oxygen radical absorbance capacity (ORAC) assay as described by Dávalos, Gómez-Cordovés, and Bartolomé (2004). ORAC values were expressed as Trolox equivalents by using the standard curve calculated for each assay and the final results were expressed in μmol equivalents of Trolox/g CS extract. All measurements were performed in triplicate.

2.11. FRAP assay

Ferric reducing antioxidant power (FRAP) was determined as described by Morales, Martin, Açar, Arribas-Lorenzo, and Gökmen (2009). Aqueous solutions of Trolox at various concentrations were used for calibration. All measurements were performed in triplicate. Results were expressed as μmol TEAC/g CS extract.

2.12. DPPH assay

DPPH radical-scavenging activity was determined as described by Morales et al. (2009). Aqueous solutions of Trolox at various concentrations were used for calibration. All measurements were performed in triplicate. Results were expressed as μmol TEAC/g CS extract.

2.13. In vitro glycation assay with BSA–glucose

BSA–glucose (BSA–Glc) assay was based on Peng, Cheng, et al. (2008). Briefly, BSA (35 mg/mL) and glucose (175 mg/mL) were dissolved in phosphate buffer (0.1 M, pH 7.4). BSA solution also contained 0.1 g/mL sodium azide. BSA solution (200 μL) was incubated with glucose solution (400 μL) at 37 °C for 21 days in the absence or the presence (100 μL) of soluble CS extracts, caffeine, or CGA (concentration of the stock solutions at 1, 5, and 10 mg/mL). In parallel, CS extracts and standards were incubated at 37 °C for 21 days in order to measure their intrinsic fluorescence. The final concentration of each reactant in the reaction medium was: 10 mg/mL for BSA, 100 mg/mL for glucose, 0.6 mg/mL for AG, and 0.14, 0.71 or 1.42 mg/mL for the different solutions of caffeine, CGA, and CS extracts. The IC_{50} values (concentration in mg/mL required to inhibit glycation by 50%) were calculated from the dose–response curves using Microsoft-Excel computer software.

2.14. In vitro glycation assay with BSA–MGO

BSA–MGO assay was performed according to Lunceford and Gugliucci (2005) with minor modifications. Briefly, BSA (35 mg/mL) and MGO (0.4 mg/mL) solutions were prepared in phosphate buffer (0.1 M, pH 7.4). Two hundred microliters of BSA solution, containing 0.1 g/mL sodium azide, was incubated with 400 μL of MGO solution. The incubation was carried out at 37 °C for 14 days in the absence or the presence (100 μL) of soluble CS extracts, caffeine, or CGA (concentration of the stock solutions at 1, 5, and 10 mg/mL). In parallel, CS extracts and standards were incubated at 37 °C for 14 days in order to measure their intrinsic fluorescence. The final concentration of each reactant in the reaction medium was 10 mg/mL for BSA, 0.23 mg/mL for MGO, 0.6 mg/mL for AG and 0.14, 0.71 and 1.42 mg/mL for the different solutions caffeine, CGA and CS extracts. The IC_{50} values were calculated from the dose–response curves using Microsoft-Excel computer software.

2.15. AGE fluorescence measurement

The measurement of fluorescent intensity of total AGEs and the intrinsic fluorescence of the CS extracts and standards after incubation were performed using a microplate spectrophotometer. The presence of total AGEs was characterized by a typical fluorescence with excitation and emission maxima at 360 and 420 nm, for BSA–Glc assay and 340 and 420 nm, for BSA–MGO assay. Percentage inhibition of AGE formation by each sample was calculated using the following equation; % inhibition = [1 − ((fluorescence of the solution with inhibitors − intrinsic fluorescence of the samples) / fluorescence of the solution without inhibitors)] × 100%.

2.16. Evaluation of direct MGO trapping capacity

Direct MGO trapping capacity was as described by Peng, Zheng, et al. (2008) with some modifications (Mesías et al., 2013). Pyridoxamine (PM) was used as the positive control. The final concentration of each reactant in the reaction medium was 0.04 mg/mL for MGO, 0.1 mg/mL for PM and a range of 0.001–0.5 mg/mL for soluble CS extracts, caffeine and CGA. Samples were incubated at 37 °C for 168 h, after that 200 μL of OPD was added. The unreacted MGO was quantified by HPLC (Shimadzu, Kyoto, JP) on the basis of the amount of the derivatized product, 2-methylquinoxaline (2-MQ). The chromatographic separation was carried out on a Mediterranean-Sea-ODS2 column (150 × 3 mm, 5 μm, Tecknokroma, Barcelona, Spain). The amounts of unreacted MGO in the samples could be determined on the basis of the ratios of peak area of 2-MQ and 5-MQ. Percentage decrease in MGO was calculated using the following equation: MGO decrease % = [(amounts of MGO in control − amounts of MGO in sample or PM solution) / amounts of MGO in control] × 100%. The IC_{50} values of samples were calculated from the dose–response curves using Microsoft-Excel computer software.

2.17. Statistical analysis

Statistical analyses were performed using the Statgraphics Centurion XV statistical program (Herndon, VA). Data were expressed as the mean value ± SD. Analysis of variance (ANOVA) and the Duncan test were applied to determine differences between means. Differences were considered to be significant at $p < 0.05$.

3. Results and discussion

Table 1 summarizes the content in dietary fiber (total, soluble and insoluble), melanoidins, chlorogenic acids, caffeine, total phenolic, antioxidant and browning of water soluble CS extracts. TPC was of 31.0 and 35.4 mg equivalents CGA/g for Arabica and Robusta CS extracts, respectively. CS has previously been considered a good source of phenolic compounds (Bresciani, Calani, Bruni, Brighenti, & Del Rio, in press). CGA content was significantly higher in Robusta CS extract (68.52 mg/g) than in Arabica CS extract (11.18 mg/g). Results of CGA are in line with

Table 1
Characterization of Arabica coffee silverskin (ACS) and Robusta coffee silverskin (RCS) extracts.

Parameters	ACS extract	RCS extract
Fiber		
Total (mg/g)	286.89 ± 19.15 a	362.18 ± 13.74b
Soluble (mg/g)	240.15 ± 19.5a	268.04 ± 6.44b
Insoluble (mg/g)	46.75 ± 0.34a	94.14 ± 7.30b
Melanoidins (mg/g)	172.67 ± 3.54a	239.46 ± 8.49b
CGAs (mg/g)	11.18	68.52
3-CGA (%)	13.43	11.97
4-CGA (%)	16.26	13.28
5-CGA (%)	57.75	38.45
Caffeine (mg/g)	30.26	33.98
Browning (A_{405}/g)	94.55 ± 1.51a	106.88 ± 0.83b
Total phenolic content (mg eq GA/g)	31.00 ± 0.24a	35.41 ± 0.42b
ABTS (μmol TEAC/g)	85.20 ± 1.91a	225.8 ± 24.8b
ORAC (μmol TEAC/g)	1194 ± 76.62a	1513 ± 9.99b
FRAP (μmol TEAC/g)	829.8 ± 38.16a	640.1 ± 39.78b
DPPH (μmol TEAC/g)	219.9 ± 4.34a	231.3 ± 4.73b

Results are expressed as mean ± SD for $n = 4$. Different letters mean significant differences.

those reported by Narita and Inouye (2012) and del Castillo et al. (2013). In a similar way, caffeine content was also higher in Robusta CS extract (33.98 mg/g) in comparison with Arabica CS extracts (30.26 mg/g). For browning determination and melanoidin content, moreover, these differences were significant. These results agree with those from Martinez-Saez et al. (2014) who described that levels of CGA, caffeine concentration, melanoidins and browning, expressed as color, were significantly greater ($p < 0.05$) in beverages made with Robusta coffee silverskin extract than Arabica.

The antioxidant capacity (radical scavenging, and hydrogen and electron donating capacities) of the CS extracts was assessed by ABTS, ORAC, DPPH and FRAP assays, showing results of 85.20, 1194, 829.8 and 219.9 μmol TEAC/g sample in the case of Arabica CS extract and 225.8, 1513, 640.1 and 231.3 μmol TEAC/g sample for Robusta CS extract. Arabica CS extract showed higher reducing power than Robusta CS extract. In contrast, Robusta CS extract showed significantly higher scavenging properties against the ABTS radical than Arabica CS extract. The results agree with those described by Napolitano et al. (2007), del Castillo et al. (2013) and Martinez-Saez et al. (2014), who found, a higher proportion of extractable antioxidants in aqueous solution for the samples of Robusta silverskin, in comparison with Arabica. The differences found in the total antioxidant capacity values in ACS and RCS extracts may be related to those detected by analyzing their components, melanoidins and CGA since, as it has been suggested, these compounds contribute to the antioxidant properties of coffee silverskin.

Fig. 1 shows the effect of CS extracts, caffeine, and chlorogenic acid on the formation of fluorescent AGEs in glycation model systems composed by BSA and glucose treated at 37 °C for 21 days. Caffeine did not affect the formation of AGEs under our particular conditions. CGA showed antiglycative capacity being its IC_{50} value of 0.4 mg/mL. Kim et al. (2011) already described the antiglycative capacity of CGA in the formation of fluorescent AGEs and crosslinking of collagen. Arabica and Robusta CS extracts showed similar and dose-dependent rates of AGE inhibition with an IC_{50} of 0.6 mg/mL for both extracts. The only significant differences ($p < 0.05$) were found at the concentration of 0.71 mg/mL where ACS showed a significantly higher antiglycative activity than RCS.

Fig. 2 represents the antiglycative capacity of the Arabica and Robusta CS extracts, caffeine, and chlorogenic acid in glycoxidation model systems (BSA–MGO) at 37 °C for 14 days. MGO was reported as a potent agent for AGE generation and modified proteins irreversibly by targeting the side chains of arginine at a much faster rate than reducing sugars (Oya et al., 1999). Caffeine (1.4 mg/mL) exerted a weak antiglycative capacity corresponding to 10% of the inhibition of the formation of fluorescent AGEs. The antiglycative capacity of CGA was notably higher in the BSA–MGO assay (IC_{50} of 0.1 mg/mL) as compared with the BSA–glucose assay. However, the antiglycative capacity of Arabica and Robusta CS extracts was lower (IC_{50} of 1.3 mg/mL) as compared with the BSA–glucose assay. Again, significant differences between both CS extracts were not observed, and they behaved in a dose-dependent manner.

AGE formation can be suppressed by inhibitors at the post-Amadori stage which can scavenge carbonyl precursors. As a consequence, the direct MGO-trapping capacity of Arabica and Robusta CS extracts was determined. Fig. 3 shows the MGO trapping ability of the CS extracts at 168 h in a range of concentrations from 0.001 to 0.5 mg/mL. Pyridoxamine (0.1 mg/mL) was used as control since it reacts with methylglyoxal to form stable adducts (Nagaraj et al., 2002). IC_{50} value for PM was 0.006 mg/mL. Caffeine, chlorogenic acid, and the Arabica and Robusta CS extracts trapped MGO in a dose-dependent manner. The lowest effectiveness was shown by the caffeine whereas only 20% of the initial MGO was decreased in the presence of 0.5 mg/mL caffeine. MGO was effectively quenched by CGA with an IC_{50} of 0.14 mg/mL. Arabica and Robusta CS extracts showed significant differences in their ability to trap MGO with an IC_{50} of 0.055 and 0.65 mg/mL for

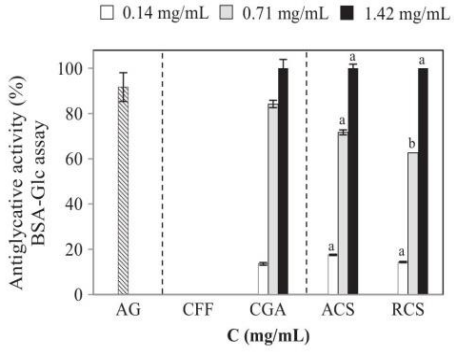

Fig. 1. Antiglycative capacity of Arabica and Robusta coffee silverskin extracts (ACS, RCS), caffeine (CFF) and chlorogenic acid (CGA) in the BSA–glucose assay. Concentrations assayed were 0.14, 0.71 and 1.42 mg/mL. Results are expressed as mean ± SD for $n = 4$. Aminoguanidine (AG) (0.6 mg/mL) showed an antiglycative activity of 91.2%. Different letters mean significant differences among the ACS and RCS extracts for a same concentration.

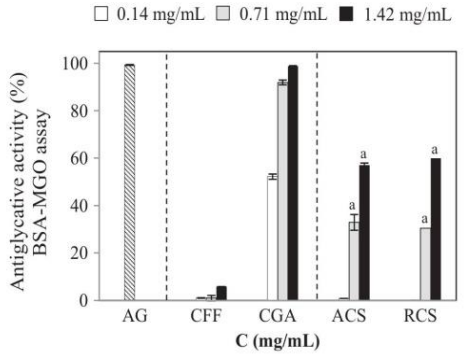

Fig. 2. Antiglycative capacity of Arabica and Robusta coffee silverskin extracts (ACS, RCS), caffeine (CFF), and chlorogenic acid (CGA) in the BSA–MGO assay. Concentrations assayed were 0.14, 0.71 and 1.42 mg/mL. Results are expressed as mean ± SD for $n = 4$. Aminoguanidine (AG) (0.6 mg/mL) showed an antiglycative activity of 99.2%.

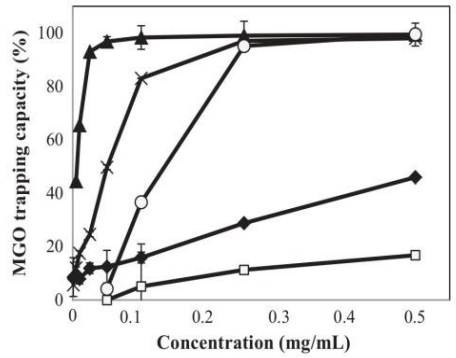

Fig. 3. Methylglyoxal trapping capacity of Arabica and Robusta coffee silverskin extracts (ACS, RCS), pyridoxamine (PM), chlorogenic acid (CGA), and caffeine (CFF) at 168 h. Results are expressed as mean ± SD for $n = 4$. PM (0.1 mg/mL) showed a MGO trapping capacity of 99.6%. ACS; RCS; PM; caffeine; CGA.

Robusta and Arabica CS extracts, respectively. Robusta CS extract was nearly 10-fold more active against MGO than Arabica CS extract.

To get more insight into the MGO trapping capacity of the extracts, kinetic evaluation for MGO-trapping capacity for 168 h at a concentration of 0.1 mg/mL was performed and compared with that of the PM (Fig. 4). PM and Robusta CS extract already trapped 50% of the initial MGO in the system at 27 and 66 h, respectively. In contrast, the Arabica CS extract only reached to trap around 14% of the MGO at 168 h, which showed a significant lower MGO-trapping capacity of this extract. The MGO-trapping capacity of the Arabica and Robusta CS extracts increased continuously during the time of incubation at 37 °C, following the same trend as to that of PM.

In the present study the antiglycative capacity measured as the inhibition of the formation of fluorescent AGEs, and mitigation of carbonyl radical stress (in terms of MGO-trapping capacity) of Arabica and Robusta CS aqueous extracts have been demonstrated. These results are in agreement with Verzelloni, Pellacani, et al. (2011) and Verzelloni, Tagliazucchi, et al. (2011) who reported that coffee contains molecules with *in vitro* antiglycative capacity. Our investigation demonstrates that antiglycative properties are also maintained in coffee by-products which may be related to phenolic compounds naturally present in coffee beans (Napolitano et al., 2007) and melanoidins formed through Maillard reaction during roasting (Delgado-Andrade & Morales, 2005). According to Borrelli et al. (2004) antioxidant compounds present in coffee can form covalent links with carbohydrates resulting in a fiber–antioxidant complex. Specifically the phenolic compounds from CS, mainly chlorogenic acid, may react with polysaccharide components forming melanoidins, which exerts the antioxidant activity (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002). According to Verzelloni, Pellacani, et al. (2011) and Verzelloni, Tagliazucchi, et al. (2011) the high molecular weight compound fraction of coffee has high antioxidant, chelating and antiglycative activities. Chlorogenic acid and fiber contents were significantly higher in Robusta CS extract as compared with Arabica CS extract.

Antiglycative effects of phenolic compounds have been previously reported. These compounds can exert their inhibition through their antioxidant properties, scavenging of free radicals, and quenching of carbonyl radical species (Delgado-Andrade & Morales, 2005; Kim & Kim, 2003; Sang et al., 2007; Wu & Yen, 2005). Wu, Hsieh, Wang, and Chen (2009) evaluated the ability of phenolic acids to inhibit glycation, concluding that these compounds were effective in the prevention of glucose-mediated protein modification, which are considered potent inhibitors of both AGE formation and the subsequent crosslinking of proteins. Among the phenolic acids studied by these authors, chlorogenic acid was considered to be one of the major antiglycative compounds, being in line with our results. In a similar way, Gugliucci, Bastos, Schulze, and Souza (2009) reported for yerba maté water extract containing chlorogenic acid and caffeic acid a high percentage of AGE fluorescence inhibition.

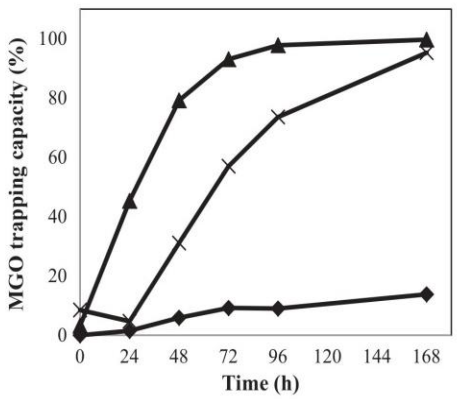

Fig. 4. Time-course MGO trapping capacity of the Arabica and Robusta coffee silverskin extracts (ACS, RCS). Results are expressed as mean ± SD for $n = 4$. Pyridoxamine (PM) (0.1 mg/mL) showed a MGO trapping capacity of 99.6%. PM; ACS; RCS.

As mentioned above, the present investigation was carried out using aqueous extracts in order to perform a cost-effective and environmentally friendly procedure, as well as being more interesting from safety and toxicological points of view for their industrial application. Several authors have evaluated the influence of the extraction process on the antioxidant capacity, concluding that water is highly efficient at extracting antioxidants. In this sense, Budryn et al. (2009) reported that both chlorogenic acids and melanoidins were found in higher amounts in aqueous extracts than in ethanolic extracts from green and roasted coffee, showing that these antioxidants were more soluble in water. In a similar way, Yen, Wang, Chang, and Duh (2005) indicated that the highest antioxidant yields in extracts were obtained with water from roasted coffee residues. Furthermore, Bravo, Monente, Juániz, De Peña, and Cid (2013) stated that water is necessary to extract more phenolic and nonphenolic antioxidants from spent coffee. Consequently, it can be supposed that the aqueous CS extracts contain the majority of the antioxidant compounds present in the CS including chlorogenic acid and, hence, it might justify the high antiglycative activity of the extracts.

Regarding caffeine, it has been shown that this compound has a low *in vitro* antioxidant activity (Somoza et al., 2003; Yen et al., 2005). In addition, caffeine has displayed a low inhibitory effect on AGE formation in *in vitro* assay systems (Nakagawa, Yokozawa, Tarasawa, Shu, & Juneja, 2002). These findings are in accordance with those found in the present study, since caffeine practically did not exhibit any antiglycative effect in the assays of BSA–glucose and BSA–MGO and a low MGO trapping capacity in comparison with that found for CS extracts. However, reactive oxygen species scavenging has been recently proposed for caffeine (Leon-Carmona & Golano, 2011).

Concerning the MGO-trapping capacity, Robusta CS extract trapped MGO more rapidly and efficiently as compared with Arabica CS extract. It might be indicated that the different compositions of the extracts influenced their MGO-trapping ability but not significantly the antiglycative capacity in the protein assays. Although FRAP and DPPH assays did not show large differences among Arabica CS and Robusta CS extracts, ABTS and ORAC exhibited a higher antioxidant capacity in the Robusta variety. In addition, despite no differences were observed in total phenolic content of the two studied samples, CGA content and specifically 3-CGA, 4-CGA and 5-CGA contents were also higher in the Robusta specie (8.2, 9.1 and 26.3 mg/g *versus* 1.5, 1.8 and 6.4 mg/g in Arabica CS), which could explain the major antiglycative capacity of this extract. On the other hand, it should be taken into account that the presence of several AGE inhibitors in an extract may have synergistic effects (Chompoo, Upadhyay, Kishimoto, Makise, & Tawata, 2011). During coffee roasting, a part of CGA is incorporated into the melanoidins (Moreira, Nunes, Domingues, & Coimbra, 2012) and the new structures may contribute to the overall antiglycative capacity of the CS extracts. Specifically, according to Rice-Evans, Miller, and Paganga (1996), phenolic compounds may have synergistic or antagonistic effects among them or with other constituents of an extract. Therefore, it can be supposed that chlorogenic acid together with other phenolic compounds, and CGA–melanoidins rich might contribute overall to the antiglycative activity of the extracts.

In spite of the antiglycative properties of the aqueous extract of coffee silverskin demonstrated in the *in vitro* assays in the present study, it cannot be assured that this effect also occur in *in vivo* experiments. According to Verzelloni, Pellacani, et al. (2011) and Verzelloni, Tagliazucchi, et al. (2011), after ingestion by humans polyphenolic compounds can be metabolized generating different metabolites in the

colon, which can or not exert inhibitory activity against AGE formation. These observations may be taken into account for future research.

4. Conclusions

The antiglycative capacity of coffee silverskin (CS) extracts, obtained from Arabica and Robusta varieties, was evaluated using different *in vitro* models. Both Arabica and Robusta CS extracts exhibited an anti-AGE capacity in BSA–glucose and BSA–MGO assays at concentrations ranging from 0.1 to 1.5 mg/mL. No significant differences among the Arabica and Robusta CS extracts were observed. On the other hand, the direct MGO-trapping assay showed that the CS extracts trapped MGO in a dose-dependent manner, but the Robusta CS extract showed a higher trapping capacity ($IC_{50} = 0.55$ mg/mL) in comparison with Arabica CS extract. Chlorogenic acid content in samples did not explain the antiglycative properties of the CS extracts, although the MGO trapping capacity was greatly related to the CGA content. The anti-AGE capacity of the samples might be related to the antioxidant capacity of the CS, explained by the phenolic compound content, especially chlorogenic acid, but also by the presence of high molecular weight polymers (melanoidins) formed through Maillard reaction during the roasting of coffee. The synergistic contribution of other constituents of the CS extracts to the antiglycative and carbonyl trapping properties *in vitro* should not be discarded. Further studies should be performed for evaluating the bioactivity of the extracts *in vivo*.

Acknowledgments

This research is funded by the Ministry of Science and Innovation (Spain) under project NATURAGE (AGL2010-17779) and CSIC-201370E027. M. Navarro is grateful to a JAE-predoc grant from CSIC. The authors wish to thank Fortaleza for supplying coffee samples for the present study.

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ANNEX 4

European Food Research and Technology

Draft Manuscript for Review

Use of almond shell as food ingredient

Journal:	*European Food Research and Technology*
Manuscript ID	EFRT-17-0278.R1
Manuscript Type:	Original paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Kacem, Imen; Unité d'Enzymes et Bioconversion, Ecole Nationale d'Ingénieurs de Sfax, ENIS, BP1173, Sfax 3038, Tunisia Martinez-Saez, Nuria; Instituto de Investigacion en Ciencias de la Alimentacion Kallel, Fatma; Unité d'Enzymes et Bioconversion, Ecole Nationale d'Ingénieurs de Sfax, ENIS, BP1173, Sfax 3038, Tunisia Chaabouni, Semia; Unité d'Enzymes et Bioconversion, Ecole Nationale d'Ingénieurs de Sfax, ENIS, BP1173, Sfax 3038, Tunisia Ben Jeddou, Khawla ; Unité d'Enzymes et Bioconversion, Ecole Nationale d'Ingénieurs de Sfax, ENIS, BP1173, Sfax 3038, Tunisia .boisset-helbert@cermav.cnrs.fr., Claire del Castillo, Dolores; Instituto de Investigacion en Ciencias de la Alimentacion
Keywords:	almond shell, antidiabetic effect, antioxidant capacity, dietary fiber, novel food ingridient, novel food

TITLE: Use of almond shell as food ingredient

AUTHORS: Imen Kacema, Nuria Martinez-Saezb, FatmaKallela, Khawla Ben Jeddoua, Claire Boisset Helbertc, Semia Ellouze Chaabounia,d, and Maria Dolores del Castillob,1

AFFILIATIONS:
aEnzymes and Bioconversion Unit, National Engineering School P.O. Box 1173-3038, Sfax University, Tunisia
bInstitute of Food Science Research (CIAL, UAM-CSIC). C/ Nicolás Cabrera, 9. Campus de Cantoblanco. Universidad Autónoma de Madrid. 28049. Madrid (Spain). www.cial.uam-csic.es.
c Service de Chromatographie Purification et analyse de polysaccharides CERMAV-CNRS, 601 rue de la Chimie, 38041 Grenoble cedex 9, France
dCommon Service Unit of Bioreactor Coupled with an Ultrafilter, National Engineering School P.O. Box 1173-3038, Sfax University, Tunisia

Corresponding author: Dr. M. Dolores del Castillo1, Tel: +34 91 0017900 Ext. 953, Fax: +34 91 0017905, e-mail: mdolores.delcastillo@csic.es.

Abstract

Almond shell is a major waste from the almond processing industry. Its feasibility as natural source of health-promoting components was examined. The by-product was fractionated under basic conditions following an easy scale-up process. The chemical composition of the recovered fraction and its antioxidant and antidiabetic properties were evaluated. Novel information regarding the chemical composition of the polysaccharides was also obtained. Almond shell is formed by lignin-carbohydrate complexes possessing antioxidant properties and capacity to inhibit α-glucosidase. According to our knowledge, this is the first time α-glucosidase inhibitory activity of a lignin-carbohydrate complex is reported. Biscuits containing non-caloric sweetener soluble (2.5%) and insoluble (5.6%) dietary fiber, natural antioxidants (1.34 mg gallic acid equivalents/g) andα-glucosidase inhibitors (1g biscuit ≈ 1mg acarbose)achieved a high sensorial score (7.2 out of 9) when almond shell was incorporated to them. The application of a fraction from almond shell containing lignin-polysaccharides complexes as food ingredient in biscuit formulations for people with particular nutritional requirements is feasible and new.

Keywords
Almond shell, antidiabetic effect, antioxidant properties, dietary fiber, novel ingredient, novel food

1. Introduction

Increased oxidative stress and inflammation processes play a major role in causing chronic diseases such as diabetes [1]. Diabetes mellitus is characterized by high blood glucose levels, resulting from insulin deficiency or functional disturbance of the receptors, with alterations in the metabolization of carbohydrates, proteins and lipids [2].
Inhibition of α-glucosidase significantly decreases postprandial hyperglycemia after the intake of a diet rich in glycemic carbohydrates, which is a key strategy in the control of diabetes [3].Acarbose, a potent α -glucosidase inhibitor, represents a new concept in the treatment of metabolic disorders, particularly type 2 diabetes and, in some countries, prediabetes. It reduces the absorption of dietary carbohydrates by reversible competitive inhibition of α-glucosidase activity, reducing post-prandial blood glucose increment and insulin response. Acarbose is considered an efficient oral

antidiabetic drug even though it produces side effects such as diarrhea and flatulence [4].Therefore, it would be meaningful to search for new glucosidase inhibitors from natural products without such side effects.

Agro-industrial by-products are considered promising opportunities, offering new biologically active and functional components for application in food and pharmaceutical industries [5].Almond shell is a major waste of the almond processing industry. This by-product has been used in the production of bioethanol [6]. However, to the best of our knowledge its application in foods as a healthy ingredient has not been previously reported. Almond shell is a thick woody endocarp, mainly composed of cellulose, hemicelluloses and lignin [7].Some polysaccharides in the cell walls of lignified plants can link to lignin to form lignin-carbohydrate complexes (LCCs). These complexes are known by their excellent anti-HIV, antiviral and pro- or anti-inflammatory activities [8]. As far as we know, there are no reports dealing with the α-glucosidase inhibitory activity of LCCs. Moreover, data on the chemical composition of extracts enriched in polysaccharides from almond shell suggest they could potentially be used as functional food ingredients with antioxidant properties [7].The development of new functional ingredients has the advantage that food manufacturers can add extra value to products the consumer is already familiar with. Biscuits could represent a potential candidate for the addition of functional ingredients, but their nutritional profile has to be improved in view of formulating functional products, reduced in sugars and caloric content. Therefore, this study aims to evaluate the effect of almond shell fractions enriched in polysaccharides and lignin on antioxidant and α-glucosidase activities; as well as their usefulness as natural food ingredients to reduce the risk or treat type 2 diabetes. Healthier biscuits have been prepared using the novel food ingredient and stevia as a natural non-caloric sweetener.

2. Materials and methods

2.1. Reagents

Fiber kit and D-glucose, mannose and fructose kit were supplied by Megazyme International Ireland Ltd. Sulfuric acid (93–98%) and ethanol (96%) were purchased from Panreac S.A. (Spain). Phenol (5%, w/v), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS)), potassium persulphate, Folin–Ciocalteu reagent and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylicacid (trolox),α-glucosidase from rat intestine and 4-methylumbelliferyl α-D-glucopyranoside (4-MUG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Water was purified using Milli-Q system. All other chemicals and reagents were of analytical grade.

2.2. Apparatus

A microplate spectrophotometer BioTekpowerWaveTM XS (BioTek Instruments, United States),a texture analyzer (LLOYD instruments, England), an activity water system (Aw Sprint TH-500, Novasina, Switzerland) and Agilent GC 6850A instrument equipped with HP-5MS capillary column were used for the analyses.

2.3. Food ingredients

The basic ingredients used to elaborate the biscuits(i.e. wheat flour, sucrose, sunflower oil, baking powder, salt, stevia powder containing 3% steviol glycosides) were purchased at specialized and certified food local markets. Food grade soy lecithin was provided by Manuel Riesgo SA (Spain).

Almond shell was collected in the city ofSfax (Tunisia), ground and sieved to small particles between 0.2 mm and 0.5 mm. Data on physicochemical characterization of almond shell is provided in Online Resource 1.

2.4. Sample preparation

2.4.1. Almond shell as novel food ingredient

Extraction was performed following the procedure described by Ebringerová et al. [7] with minor modifications to obtain fractions with antioxidant capacity and a high polysaccharide content. Ground almond shell was treated with 5% NaOH (1:10 w/v) at 60°C for 1h, vacuum filtered in a büshi funnel using cellulose filter paper, added with three volumes of isopropanol and incubated overnight at 4°C.Finally, the fraction was isolated by decreasing the pH to 7by adding concentrated acetic acid and centrifuging at 4500 rpm for 15 min at 20 °C. The isolate fraction was exhaustively dialyzed against distilled water under magnetic stirring for three days at 4°Cand then freeze-dried. The powder was stored in sealed plastic bags at 4 °C until use.
The obtained alkali-extracted almond shell fraction (AEASF) was dissolved in water (1:10 w/v) and centrifuged at 4000 rpm for 10 min at 20°C. The supernatant (water soluble fraction, WSF)and precipitate (water insoluble fraction, WIF), were recovered. WIF was washed with water (1:10 w/v) and centrifuged. Water soluble fractions were then mixed. WSF and WIF were frozen at -20°C, freeze-dried and stored until analysis. WSF in a concentration of 1mg/ml was further fractionated by ultrafiltration using filter devices with a molecular weight cut-off of 3 kDa (Millipore), following the instructions of the manufacturer. Fractions containing water soluble compounds with higher (HMW) and lower (LMW) molecular weight than 3 kDa were frozen at – 20°C and freeze-dried. Powdered samples (AEASF, WIF, WSF, HMW and LMW)were stored under dry conditions until analysis. Samples preparations were performed in triplicate. Yields are expressed as percentage (%).

2.4.2. Food preparation

Biscuits with AEASF as food ingredient (B, C, E and F) and those without AEASF (controls, A and D) were formulated as indicated in Table 1.The dough was prepared by mixing salt, baking powder and sugar or stevia. Mineral water at room temperature was added to the dry mixture and thoroughly blended to obtain a homogeneous mixture. Lecithin and oil were mixed in a separate bowl and then added to the mixture. Finally, flour was added gradually to the mixture, and the dough was kneaded to obtain homogeneous, elastic and slightly sticky dough. The dough was allowed to rest for 30 min and shaped into discs with a 4 cm diameter and 0.8 cm thickness. The surface of each biscuit was punctured several times using a fork to prevent puffing. In the formulations with AEASF, it was combined with the flour and added as described above. The biscuits were baked at 190°C for 20 min in the oven. Two sets of 4 biscuits were baked in duplicate (n=8). The biscuits were placed in the center of the tray forming a square, in order to reduce variability during baking. The biscuits were cooled to room temperature prior to analysis (18–20 °C).

2.5. Physico-chemical and nutritional characterization of the novel ingredient (AEASF) and food formulations

2.5.1. Free mannose, fructose and glucose

Mannose, fructose and glucose contents were determined in WSF, HMW and LMW fractions using an enzymatic kit following the manufacturer's instructions (D-Mannose-D-Fructose-D-Glucose Assay procedure, Megazyme International Ireland, Ireland). The method was adapted to a micromethod format. Results were expressed as %. The analysis was performed in triplicate.

2.5.2. Total carbohydrates

Carbohydrate content was determined in WSF and its corresponding HMW and LMW fractions using the phenol–sulfuric acid method described by Musako et al. [9]. The color reaction was initiated by mixing 100 µl of samples with 300 µl of concentrated sulfuric acid (93–98%) and followed with the addition of 90 µl of phenol (5%, w/v) in a glass flask. The reaction mixture was kept at 90°C for 5 min. After cooling the samples to room temperature, the absorbance was measured at 490 nm. A calibration curve was constructed using glucose (0.1–0.4 mg/ml). Reagent and sample blanks were also prepared and analyzed in each set of samples. All measurements were performed in triplicate and results were expressed as %.

2.5.3. Dietary fiber

Dietary fibre (DF) content (insoluble (IDF), soluble (SDF) and total (TDF)) was determined in AEASF and its soluble (WSF) and insoluble (WIF) fractions using the Total Dietary Fiber Assay Kit (Megazyme International Ireland, Ireland) as indicated in the manufacturer's instructions, and based on an enzymatic–gravimetric method. Results were expressed as %.The analysis was performed in triplicate.

2.5.4. Monosaccharide composition of polysaccharides extracted from almond shell

The elemental monosaccharide composition of the polysaccharide extracted from WSF, LMW and HMW fractions was analyzed by gas chromatography-flame ionization detector (GC-FID) as described by Ben Jeddou et al.[10].

2.5.5. Lignin content

The content in lignin was measured on WIF, WSF, HMW and LMW fractions from the AEASF. On the basis of the standard T222 om-11, 1g (m0) of oven-dry-matter was placed in a 1 L flask with 15.0 mL of 72% sulphuric acid and kept at 20°C for 1 h in a thermostatic water-bath. After this, 575 mL of distilled water were added and the mixture was brought to boiling for 4 h under reflux. The insoluble fraction was recovered in a tarred fritted glass filter (m1) of porosity No. 3 and washed with hot water until neutral pH. The set filter-lignin was oven-dried at 105°C for 24 h, cooled in a desiccator and finally weighed (m2), determining the acid insoluble lignin content (% in dry basis) as:

$$Acidinsolublelignin(\%)=(m\_2-m\_1)/m\_0 \times 100$$

The filtrate obtained from the crucible was kept for soluble lignin estimation, which was measured spectrophotometrically at 280 nm. Acid-soluble lignin was determined according to TAPPI UM 250 standards. It can be estimated through the following expression:

$$\text{Acid soluble lignin (\%)} = \{(\text{Absorbance at 280 nm} \times \text{Dilution factor}/20) \times 100\}/(1000 \times m0)$$

2.5.6.Total phenolic content

Folin–Ciocalteu adapted to a micromethod format [11] was selected to determine the total phenolic content (TPC) in theWSF, HMW and LMW fractions. The reaction was initiated by mixing 10 µl of sample with 150 µl of freshly prepared Folin–Ciocalteu solution. The mixture was equilibrated for 3 min at room temperature and then 50 µl of sodium bicarbonate solution were added. The reaction was followed for 120 min at 37 °C by measuring absorbance at 735 nm. Sample blank and reagent blank were also analyzed in each set of samples. A gallic acid calibration curve was used for quantification (0.1-1mg/ml). Results were expressed as mg gallic acid equivalents (GAE)/g sample. All measurements were performed in triplicate.

2.6. Biological activities of the novel ingredient (AEASF) and food formulations

2.6.1. Total antioxidant capacity

Antioxidant capacity of the soluble fractions from the AEASF (WSF, LMW and HMW) and the biscuits formulations (A, B, C, D, E and F)was determined by ABTS radical scavenging activity measured as described by Oki et al. [12]. Previously, biscuits were dissolved in water (100 mg/ml) and centrifuged. Then, supernatants were diluted with PBS (5mM, pH7.4).
Two and a half mL of 7 mM ABTS•+ aqueous solution were mixed with 44µl of 140 mM potassium persulfate. The mixture was then allowed to stand in the dark for 16 h at room temperature. The working solution of the radical ABTS•+ was prepared by diluting the stock solution 1:75 (v/v) in 5 mmol/L sodium phosphate buffer pH 7.4 to yield an absorbance value of 0.7 ± 0.02 at 734 nm. The working solution of ABTS•+(270µl) was added to 30 µl of sample solution in a microplate. Absorbance was then measured at 734 nm for 10 min at 30°C with measurements every 2 min. After 5 min, the reaction was complete. Trolox (0.15–2 µmol/L) was used as standard and results were expressed as the %of inhibition, IC50 and µmol trolox equivalents (eq.)/g sample. All measurements were performed in triplicate.

The original QUENCHER assay (direct ABTS) was also performed on the insoluble fraction of the AEASF (WIF) by measuring the quenching activity of the ABTS•+reagent as detailed byAçar et al. [13] for insoluble fractions. WIF was first diluted 10 folds in cellulose and then 10 mg of this mixture were weighed. The reaction was started by adding 10 mL of ABTS•+. The mixture was vortexed for 1 min and placed on a rotator in the dark. After mixing for 30 min at 37°C, the sample was centrifuged at 5000 rpm for 2 min. The absorbance of clear supernatant was measured at 734 nm. The decrease in color was correlated to the antioxidant concentration. Trolox (0.2–2 mmol/L) was used to calculate overall antioxidant capacity. Results were expressed as mmol trolox eq./g WIF. All measurements were performed in triplicate.

2.6.2.Alpha-glucosidase inhibitory activity assay

This assay was performed on the soluble fractions of the AEASF (WSF, LMW and HMW) and the biscuits formulations (A-F). Alpha-glucosidase enzyme was previously extracted to the assay. Briefly, 100 mg of rat intestine powder were dissolved in 3 ml of NaCl (0.9%), sonicated in an ice bath for 6 min and then centrifuged at 10000 g for 30 min. The supernatant containing the enzyme was stored in the freezer. In a 96-well microplate, 100µl of sample dissolved in PBS 100 mM (pH 6.9) were mixed with 100µl of α-glucosidase (diluted 1/10) and 100µl of 4-MUG (2mM). Fluorescence was then monitored at an excitation wavelength of 360 nm and an emission wavelength of 460 nm for 30 min at 37°C. Blank of sample and negative control (buffer, enzyme and 4-MUG) were included. Acarbose was used as a positive control. The percentage of α-glucosidase inhibition was calculated using the equation:
"α-glucosidase inhibition (%)= " ("F" _"nc" "-" "F" _"s")/"F" _"n.c" " x 100"
Where Fn.c. and Fs are the fluorescence of the negative control and sample, respectively. All measurements were performed in triplicate.

2.7. Quality attributes of the biscuits

2.7.1. Water activity and Ph

The water activity (Aw) of biscuits (A-F) was measured by a water activity meter. The ground biscuit was placed in the specimen holder of the device to record its water activity. The analysis was performed in duplicate and the mean value was reported.

For the pH, 1 g of ground biscuit was mixed with 50 mL of deionized water and vortexed for 5 min. The mixture was held at room temperature for 1 h to separate solid and liquid phases. After carefully removing the supernatant layer, pH was measured using a pH meter. The analysis was carried out in duplicate.

2.7.2. Color and texture analyses

Color parameters were expressed according to CIE L*a*b* scale [14]. Measurements were made using the Hunter Lab system with a colorimeter (Minolta CR-300, Japan). Four independent measurements of a* (redness), b* (yellowness) and L* (lightness) parameters were carried out on the top and bottom areas of the biscuits (A-F).
The hardness of the biscuits (A-F) was measured using a texture analyzer. Results were expressed as newton (N). The analysis was carried out in duplicate.

2.8. Sensorial analysis of the biscuits

Sensory evaluations of the biscuits (A-F) were carried out. Ten pre-trained panelists participated in the sensory tests. Biscuits with 6-digit number codes were randomly presented to panelists. A 9-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, 9 = liked extremely) was used to evaluate the biscuits for color, flavor, crispness, hardness, mouth feel and overall acceptability. Panelists were asked to swallow samples and to rinse their mouths with water between samples. Five and higher scores of overall acceptability were considered as acceptable in this study.

2.9. Statistical analysis

Data were expressed as mean ± standard deviation (SD). The data were then analyzed using Duncan's method for significance tests using the software SPSS Version 13.0 (IBM® SPSS® Statistics, USA).

3. Results and discussion

3.1. Ingredient characterization

3.1.1.Chemical composition of AEASF

The extraction yield of the AEASF was 9.6±1.5%. This yield was lower than that obtained by Ebringerová et al. [7] and it may be related to the botanical origin of almond tree. An economical extraction and purification of process has been used. However, the extraction yield was achieved. The combined use of low intensity heat (60ºC) with other unconventional technologies such as ultrasound and microwave-assisted extraction, high pressure combined with thermal processing, supercritical Carbon Dioxide (SC-CO2), pulsed electric fields assisted processing to improve extraction yield of AEASF or subcritical water extraction may be investigated in the future. These approaches have been proposed as promising technologies that can be useful for extraction of glucosinolates and isothiocyanates from natural sources. They are difficult to isolate because their high reactivy. In addition, their hydrolysates are believed to be toxic and anti-nutritional for animals. Non-conventional techniques seem to ensure high extraction yields and low degradation of them [15].

Table 2(a) shows DF values corresponding to AEASF, WSF and WIF. AEASF under the conditions hereby described showed a quantity of TDF of 43.2% (w/w). The SDF content (25%) was greater than the IDF content (18.28%), with an IDF/SDF ratio of 1:1.4.DF was also found in the two fractions isolated from AEASF. WSF contained≈ 40% TDF and SDF was three times higher than IDF. WIF, accounting for 22.1±1.4%of the AEASF, was mainly composed of IDF (81±0.2). Almond

shell can be considered a natural abundant source of both SDF and IDF. SDFs have been linked to reduced cholesterol in the blood and decreased intestinal absorption of glucose, whereas IDFs are associated with water absorption and intestinal regulation [16]. These physiological effects of SDFs and IDFs depend on their structural and physical properties, which can result in behavior such as hydration, swelling and enzymatic attack [16].

Total carbohydrate content, free glycemic sugars and TPC of WSF and the corresponding HMW and LMW fractions were also evaluated. WSF was rich in carbohydrates (44.5±5%). HMW (44.5% of the WSF) presented 19.5±3.2% carbohydrates and LMW fraction (55.5% of the WSF) 25±2.6% carbohydrates. No free simple sugars (fructose, glucose and mannose) were found in the isolated fractions (WSF, HMW and LMW), indicating that ingredients with a low glycemic index were obtained. TPC of the WSF, HMW and LMW fractions were 47.6±1.8, 31.7±0.4 and 21.8±0.6mg GAE/g, respectively. These values were in the range reported by Sfahlan et al. [17], who obtained TPC values in almond shell ranging from 18.4 to 62.7 mg GAE/g. Moreover, these values obtained in this study were higher than those quantified in hazelnuts kernel which varied from 491.2 mg GAE/ kg to 1700.4 mg GAE/ kg depending on the cultivar and environmental conditions [18].

The monosaccharide composition of the WSF, HMW and LMW fractions was that illustrated in Table 2(b). The monosaccharide composition showed that the WSF is mainly composed of glucose (14.7%) and xylose (13.9%) ($p>0.05$), followed by arabinose and galactose ($p>0.05$) and finally, by galacturonic acid and rhamnoses ($p>0.05$). These results are in agreement with others studies which reported that the water soluble fraction of almond shell treated with 5% NaOH during 60 min is composed of xylan and pectin polymers. It was also reported that the water insoluble fraction is composed of xylan with low pectin contamination [7].

The monosaccharide composition of the HMW fraction showed that xylose (9.1%) was the significantly highest sugar ($p<0.05$), followed by galactose (4.7%) and arabinose (3.5%). Glucose, rhamnose and galacturonic acid were significantly lower ($p<0.05$). On the basis of the results, it can be concluded that xylose was present in the backbone and some residues of galactose, arabinose, glucose, rhamnose and galacturonic acid might be linked in the position of branched structure in the HMW fraction. As shown in Table 2(b), the LMW fraction is mainly composed of glucose (13.5%), followed by significantly smaller amounts ($p<0.05$) of xylose and arabinose. Galacturonic acid, galactose and rhamnose ($p>0.05$) were present in the lowest quantities. All these results demonstrate the heterogeneity of these polysaccharides.

Regarding the lignin composition, results showed values of 2.5±0.1%, 8.7±0.8%, 11.4±0.5% and 0.4±0.04% for the WIF, WSF, HMW and LMW fractions, respectively. Lignin is considered as non-carbohydrate DF and a complex chemical compound usually derived from wood and some algae as well as an integral part of the secondary cell walls of plants [19].All these results implied that the obtained ingredient is a lignin-carbohydrates complex in which the relative high contents of carbohydrate constituents make hydrophobic lignin highly water-soluble [20].

3.1.2. Biological activity of the novel ingredient

3.1.2.1. Total antioxidant capacity

Figure 1(a) shows the antioxidant capacity of almond shell fractions (WSF, HMW and LMW fractions). All samples presented antioxidant character. The IC50 values for WSF, HMW and LMW fractions were 0.21, 0.22 and 0.86 mg/ml, respectively, corresponding to 786.8,

768 and 192.9 µmoltroloxeq./g, respectively. All the results described above indicated that the active substances (WSF, HMW and LMW fractions) comprised lignin as well as carbohydrate, had antioxidant capacity. Previous reports showed that lignins are hindered phenolic polymers which have strong antioxidant properties [21]. Carbohydrates were also proved to exhibit free radical scavenging activities [10]. Therefore, the potential antioxidant capacity of these fractions may be due to the supply of hydrogen by the carbohydrate or lignin constituents, which combine with radicals and forms a stable radical to terminate the radical chain reaction. Basing on our results, the antioxidant power of WSF should be attributed to its HMW fraction, which contained high lignin content. Lignin seems to be major contributor to the overall antioxidant character found for WSF. The total antioxidant capacity of WSF was higher than that previously reported for hazelnut kernels (1682.5µmoltrolox eq./kg) [18].

WIF showed an overall antioxidant capacity of 1530 mmol trolox eq./g. Results indicate that almond shell is a good source of insoluble antioxidant DF. The antioxidant character of this polymer may be closely associated to the high amount of xylanin its structure [7]. The WIF antioxidant property may also be ascribed to lignin [20] which is present in 2.5%.On the other hand, phenolic compounds may be bound to the structure of polysaccharides and lignin enhancing the antioxidant properties of the complex [22].

The transportation of dietary antioxidants through the gastrointestinal tract has been described as an essential function of DF [23]. Polyphenols linked to DF may be released in the colon by the action of bacterial microbiota, producing bioactive metabolites and an antioxidant environment, thereby reducing the risk of gastrointestinal diseases associated with oxidative stress and inflammation. The presence of DF and polyphenols has also been reported to decrease the glycemic index [24].

3.1.2.2. Inhibition of intestinal α-glucosidase activity *in vitro*

The effect of the novel ingredient (AEASF) on α-glucosidase activity is shown in Figure 2(a). The three samples (WSF, HMW and LMW fractions) inhibited the activity of this enzyme. IC50values were of 1.5, 1.2 and 7.9 mg/ml, corresponding to 23.2, 30.3 and 4.5 mg acarbose eq./g for the WSF, HMW and LMW fractions, respectively. Results indicated that the HMW fraction is composed of more efficient inhibitors of the enzyme than the LMW fraction. This activity may be ascribed to both lignin-carbohydrate complex and phenols. Polyphenols possess the ability to bind to the active protein pocket of the α-glucosidase enzyme [25]. Several studies suggest that plant polyphenols act as inhibitors of carbohydrate hydrolyzing enzymes [26]. Carbohydrates were also proved to possess α-glucosidase inhibitory activity [27].Therefore, the strong potential α-glucosidase inhibitory activity may be due to both effect of carbohydrates and lignin containing polyphenols. As far as we know, there is no report to date, describing the α-glucosidase inhibitory activity of a lignin-carbohydrate polymer.

The capacity of the HMW fraction to inhibit the activity of α-glucosidase (90%) was higher than that reported for wheat, buckwheat, corn and oats (18–31%) [28], and was comparable to that found for sorghum (95%). Results also indicated that almond shell is source of α-glucosidase inhibitors. Therefore, it can be incorporated into food as a potential antidiabetic agent. To the best of our knowledge, this is the first time that this property is associated with fractions recovered from this agronomical by-product.

3.2. Characterization of biscuits

3.2.1. DF composition and bioactivity properties

The DF content of the different biscuits (A-F) is shown in table 3. The highest values were found for C and F biscuit formulations (15.4% and 17.7% TDF) containing stevia and sucrose, respectively, and both, AEASF as ingredient source of DF (43%). As expected, the lowest amounts of fiber were reported by A (sucrose) and D (stevia) control biscuits (4.0% and 4.1%). According to the nutritional claims approved by the European Commission Regulation (EU) No 1924/2006 [29] relative to fiber content, new biscuit formulations containing AEASF fiber (B, C, E and F)could be classified within "high fiber content" nutritional claim (≥ 6 g of fiber per 100 g).The incorporation of the novel ingredient (AEASF) to the biscuit formulation significantly ($p<0.05$) increased the amount of TDF above 6% and the proportion of IDF. The SDF/IDF ratios were of 1:1.7 and 1:1.5, for sucrose (A) and stevia (B) control biscuits, 1:2.2 for B and E biscuits, and 1:4.6 and 1:4.1 for C and F, respectively. The soluble/insoluble fiber ratio has been established at 1:3 [30], which in concordance with our findings. Recommendations with regard to DF consumption differ depending on the regulatory body. For instance, the World Health Organization (WHO) suggests a daily consumption of 27–40 g of TDF. However, the American Dietetic Association (ADA) recommends an intake between 20 and 30 g of fiber/day, of which 3–10 g should be SDF (15–30% soluble fiber).The consumption of a portion (≈ 45 g) of "high fiber content" biscuits supplemented with AEASF (B, C, E and F biscutis) can provide 2.8 g, 6.9 g, 3.6 g and 7.9 g of TDF and 0.86 g, 1.2 g, 1.1 g and 1.53 g of SDF, respectively. Therefore, a moderate consumption (5 units/ day) of the novel "high fiber content" biscuit formulations (F) made with stevia and AEASF fiber provides 23% of the daily average amount of TDF recommended by the WHO and 24% of the SDF recommended by the ADA.

Addition of AEASF significantly increased ($p<0.05$) the TPC of the biscuits (table 3), achieving the highest values the C and F biscuits. No significant differences in TPC were found between control biscuits (A and D) ($p>0.05$) and among the biscuits supplemented with the same percentage of fiber (B and E ($p>0.05$); C and F ($p>0.05$)). Thus, supplementation of novel biscuits with AEASF fiber not only improves the balance of DF of the food formulations but also their phenolic content.

Antioxidants and α-glucosidase inhibitors may be degraded during the baking process. No data on the thermal stability of biocompounds present in AEASF have been previously reported. Biscuit formulations containing this novel ingredient (B, C, E and F) showed significantly higher ($p<0.05$) antioxidant capacity than control biscuits (A and D) (Figure 1 (b) and (c)). The antioxidant capacity values of the biscuits containing equal amounts of AEASF (B and E; C and F) were of the same order of magnitude ($p>0.05$). Biscuits with the highest DF content (C and F) presented the greatest antioxidant capacity. These results fit with the TPC above described and support the stability of antioxidant compounds to the thermal treatment.

As can be observed in figure 2 (b) and (c), the α-glucosidase inhibitors in the novel biscuits resisted the baking process. The "high fiber content" biscuits (C, 15.4% and F, 17.6%) corresponding to the highest concentration of AEASF showed the highest inhibitory potential against the activity of α-glucosidase. According to our calculations, the intake of 1 g of biscuit may correspond to 1 mg of acarbose and moderate consumption of these novel biscuits (5 units/day) may exert an antidiabetic effect similar to that of 50 mg acarbose. Novel biscuits made using ingredients recovered from almond shell (AEASF) present a great potential

as functional and/or medical food since they may be useful in reducing the risk and treating chronic metabolic disorders related to the metabolism of carbohydrates and oxidative stress such as type 2diabetes.

3.2.2. Quality parameters

The measurement of Aw is crucial considering the development of a food product. It can be used for the determination of shelf-life and it is an analysis of quality control [31]. As shown in Table 3, sucrose replacement by stevia increased Aw. These results agree with others previously reported [32, 33]. However, the increase of almond shell DF in the formulation of biscuits slightly decreased their Aw, which is in concordance with those results described by Garcia-Serna et al. [33] who observed less moisture content when coffee silverskin was incorporated as DF in biscuits. This Aw decrease may be attributed to the greater ability to strongly bind water of the soluble and insoluble fibers present in almond shell compared to wheat flour and therefore lower water availability in the biscuits. Addition of AEASF may enhance the quality of dietary biscuits made with stevia. Similar results reported by Prokopov et al. [34], showed that adding 10% and 20% cabbage leaf power rich of dietary fiber to sponge cake, increased water binding and thereby water retention power. Aw below 0.5 limits the growth of all microorganisms and chemical degradative reactions in food are widely decreased [35]. Then, all the tested biscuits containing sucrose or stevia and with or without almond shell DF, can be considered microbiological safe. Previous studies recognize the use of dietary fiber as an aid in delaying the aging of bakery products [34].

The demand for health-oriented products that containing high fiber, natural antioxidants, and low calorie content; such as, those proposed in the present article is increasing because of their beneficial health effects [34]. The replacement of sugar by stevia scarcely decreased pH values of the biscuit formulations (Table 3) unlike those recent results found by others authors [32], who associate steviosides with increase of pH in biscuits. In contrast, supplementation with AEASF fiber significantly ($p<0.05$) increased the pH (≥7).Our results are in accordance with those pH values observed for biscuits, which have been described to range from 7.0 to 7.3 [36]. These values are suitable for stability or keeping qualities due to the fats being more readily attacked and broken down in an acid medium than in alkaline medium [37].

Sugar replacement significantly increased ($p<0.05$) L* and increased a*, color parameters (Table 3). The addition of AEASF to the biscuit formulation significantly affected their color profile (online resource 2). L* and b*parameters significantly ($p<0.05$) decreased in a dose-dependent manner, while a* increased with the addition of DF (Table 3). These results are in line with those previously reported by Garcia-Serna et al. [33].These findings support the potential of almond shell components as natural coloring.

Texture analysis of biscuits is shown in Table 3. Sucrose biscuits were harder than those made with stevia [32, 33, 38]. Addition of AEASF significantly increased ($p<0.05$) the hardness of biscuits in a dose-dependent manner. These mechanical changes are related to an increase in fibers and carbohydrates and to reduced gluten development capacity. Popov-Raljic et al. [39] previously reported that the nature of DF used in biscuits affects their rheological properties, water activity, viscosity and sensorial characteristics such as color.

According to our results, almond shell incorporated as DF in combination with stevia produces dietary biscuits with acceptable quality properties.

3.2.3. Sensorial analysis

All biscuits prepared hereby were evaluated for their color, flavor, crispness, hardness, mouthfeel and overall acceptability using 9-point hedonic scale (Table 4). According to the data, both the sweetener and the DF content have influence on the sensorial analysis of the biscuits. Regarding the use of stevia as sweetener, can be observed crispness was significantly ($p<0.05$) scored lower (D, 5.7) than the biscuit containing sucrose (A, 6.5) and overall acceptability decreased (F, 6.1) when compared to the respective biscuit made with sucrose (C, 7.1). The other attributes were not affected by the replacement of sucrose by stevia. As previously reported, fructoligosacharides (FOS) presents slightly sweetness and could enhance taste of the biscuits by masking negative off-flavors from stevia and therefore, to improve the overall acceptance [40]. As far as AEASF fiber addition is concerned, biscuits were less appreciated for the color, hardness and mouth feel attributes when high amount of AEASF was added (C and F biscuits) but more appreciated for the crispness. Similar results demonstrated by Prokopov et al. [34], showed that the addition of 10% and 20% cabbage leaf powder changed the sensory cakes characteristics, which became less appreciated for the sensation for sweetness and the color.

Moreover, there were no significant differences in the flavor for all biscuits, contrary to what was reported by Prokopov et al. [34], who showed that the sponge cake made with cabbage leaf powder has a strongly expressed odor specific towards the sample odor. Moreover, those "high fiber content" biscuits supplemented with AEASF in their formulations (B, 6% DF and E, 8% DF) achieved the highest scores for all sensorial parameters.

In the overall acceptance, all evaluated biscuits resulted in formulations with acceptable sensory characteristics. The modifications of the control biscuit composition did not significantly impair overall acceptability of the final products. However, the highest scores were attributed to biscuits (B and E) prepared with sucrose and stevia and supplemented with AEASF with 6% and 8% of DF, respectively (B, 7.6 and E, 7.2). The lowest values were for the control biscuits (A, 7 and D, 6.4). Therefore, we could consider AEASF highly-accepted ingredient to include in innovative biscuits since diary baked products were obtained according with consumers' preferences.

4. Conclusion

Fractions recovered from almond shell following the procedures hereby described have a great added value due to their potential to prevent and/or treat diabetes as a diet-related chronic disease. Biscuits made with the almond shell contain SDF and IDF, natural antioxidants and inhibitors of α-glucosidase enzyme and, in addition, they present acceptable sensorial properties according to consumers' preferences.

Acknowledgements

The present research was funded by AGL2014-57239-R project and the Ministry of Higher Education, Tunisia. Martinez-Saez, N. thanks the Autonomous University of Madrid (U.A.M) for her FPI-predoc fellowship.

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FIGURE CAPTIONS

Figure 1.Concentration-response curves of total antioxidant capacity as the percentage (%) inhibition of the radical ABTS•+ of the (a) fractions recovered from almond shell (WSF, water soluble fraction; HMW, water soluble compounds with molecular weight higher than 3 kDa; LMW, water soluble compounds with

molecular weight lower than 3 kDa), (b) biscuits containing sucrose (A, control) and AEASF fiber (Band C, “high fiber content”), and (c) biscuits containing stevia as sweetener (D, control) and AEASF fiber (Eand F, “high fiber content”). Data are presented as mean ± SD.

Figure 2.Inhibitory effect on alpha-glucosidase activity of the (a) fractions recovered from almond shell (WSF, water soluble fraction; HMW, water soluble compounds with molecular weight higher than 3 kDa; LMW, water soluble compounds with molecular weight lower than 3 kDa), (b) biscuits containing sucrose (A, control) and AEASF fiber (B and C, “high fiber content”), and (c) biscuits containing stevia as sweetener (D, control) and AEASF fiber (E and F, “high fiber content”).Data are presented as mean ± SD.

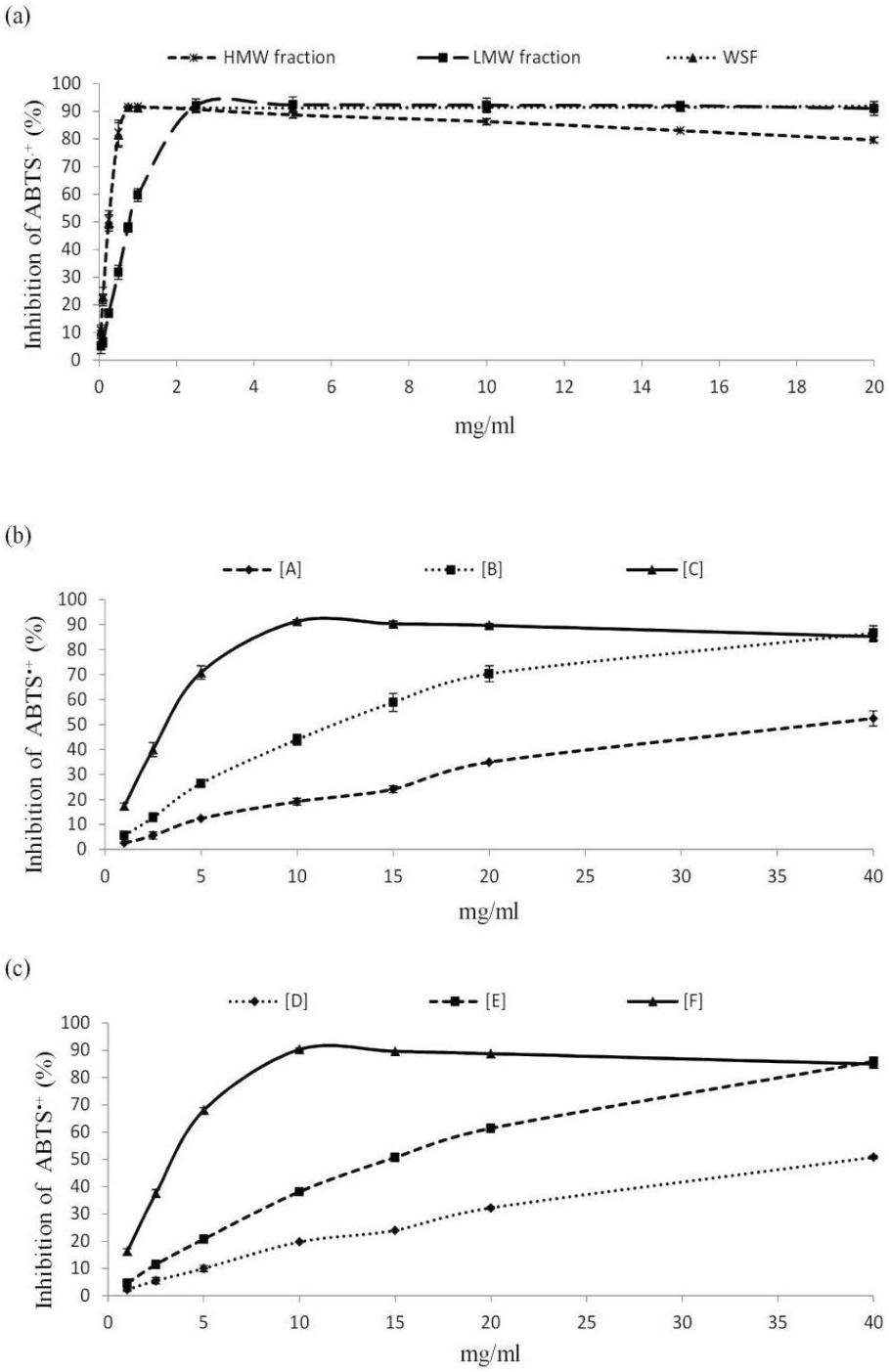
(a)
HMW fraction
LMW fraction
WSF
Inhibition of ABTS·+ (%)
mg/ml
(b)
[A]
[B]
[C]
Inhibition of ABTS·+ (%)
mg/ml
(c)
[D]
[E]
[F]
Inhibition of ABTS·+ (%)
mg/ml

Figure 1.

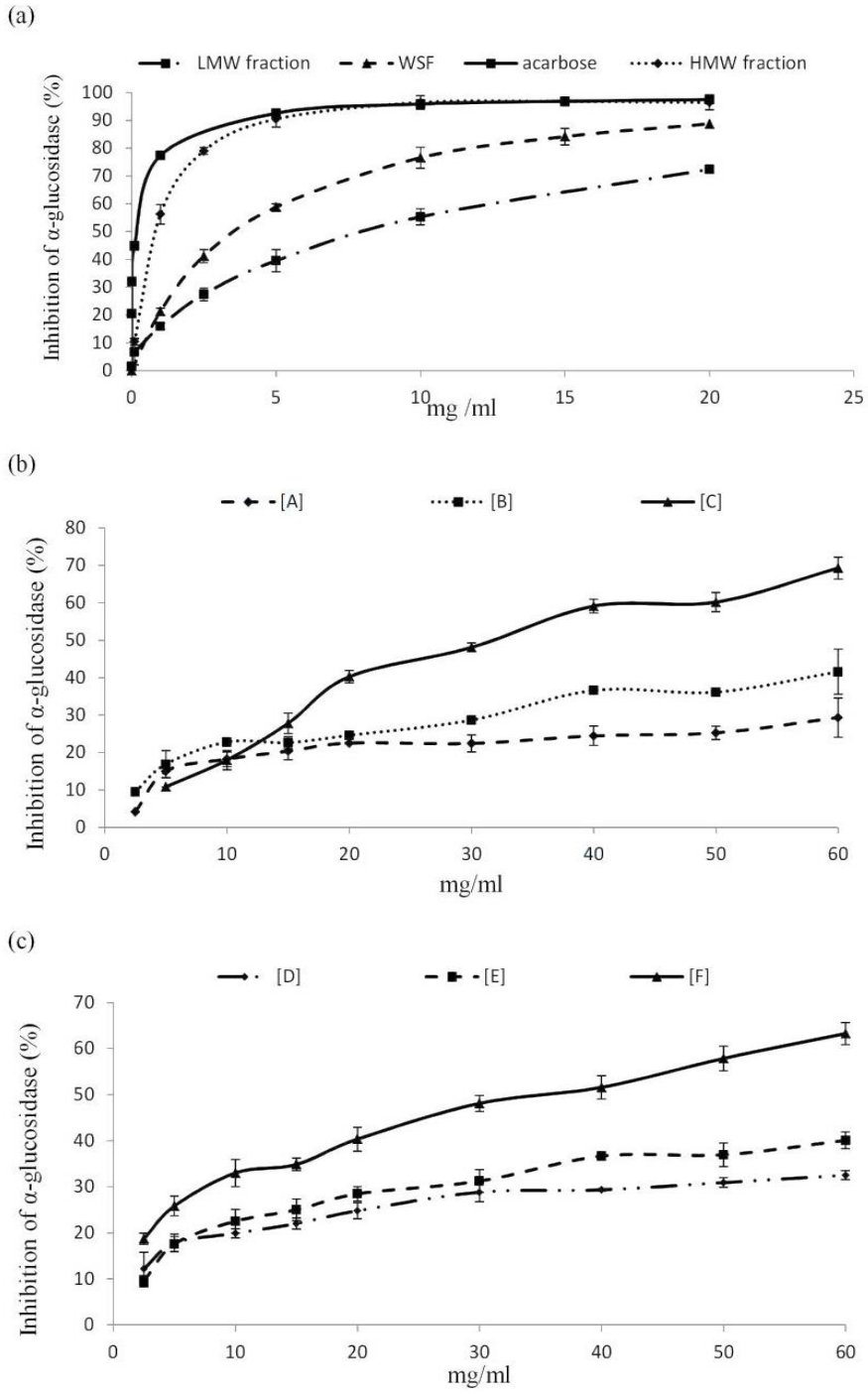
(a)
LMW fraction
WSF
acarbose
HMW fraction
Inhibition of α-glucosidase (%)
mg /ml
(b)
[A]
[B]
[C]
Inhibition of α-glucosidase (%)
mg/ml
(c)
[D]
[E]
[F]
Inhibition of α-glucosidase (%)
mg/ml

Figure 2.

Table 1. Food formulations: A, control biscuit using sucrose as sweetener; B and C, sucrose biscuits containing alkali-extracted almond shell (AEASF, 3.9% and 15.3%, respectively) as source of dietary fiber; D, control biscuit using stevia as sweetener; E and F, stevia biscuits containing AEASF (3% and 14.7%, respectively).

Ingredients (g)	Biscuit formulations					
	A	B	C	D	E	F
Wheat flour	56.00	52.13	40.70	65.85	62.84	51.09
Water	20.00	20.00	20.00	23.50	23.51	23.51
Sunflower oil	7.75	7.75	7.75	9.11	9.11	9.11
Baking powder	0.55	0.55	0.55	0.65	0.65	0.65
Salt	0.37	0.37	0.37	0.43	0.43	0.43
Lecithin	0.33	0.33	0.33	0.39	0.39	0.39
Sucrose	15.00	15.00	15.00	-	-	-
Stevia	-	-	-	0.07	0.07	0.07
AEASF	-	3.87	15.30	-	3	14.75
Total	100	100	100	100	100	100

Table 2. (a) Content of total (TDF), insoluble (IDF) and soluble (SDF) dietary fiber of the alkali-extracted almond shell (AEASF) and its soluble (WSF) and insoluble (WIF) fractions, and (b) monosaccharide composition of the water soluble fraction (WSF) and its corresponding high (HMW) and low (LMW) molecular weight fractions. Results were expressed as %.

(a)

	TDF (%)	IDF (%)	SDF (%)
AEASF	43.24 ± 0.76^{b}	18.28 ± 0.49^{b}	24.96 ± 1.91^{b}
WSF	39.88 ± 0.26^{a}	9.73 ± 0.81^{a}	30.16 ± 0.48^{c}
WIF	87.55 ± 0.40^{c}	80.95 ± 0.21^{c}	6.60 ± 0.58^{a}

Data are presented as mean ± SD. Different letters indicate significant differences ($p<0.05$) between the samples of the same column.

(b)

Monosaccharide (%)	WSF	HMW	LMW
Arabinose	7.13 ± 0.49^{b}	3.45 ± 0.62^{b}	3.68 ± 0.18^{b}
Galactose	5.88 ± 1.57^{b}	4.72 ± 0.73^{c}	1.16 ± 1.18^{a}
Glucose	14.69 ± 1.44^{c}	1.15 ± 0.15^{a}	13.54 ± 1.82^{c}
Xylose	13.92 ± 1.24^{c}	9.07 ± 1.41^{d}	4.85 ± 0.24^{b}
Rhamnose	0.91 ± 0.08^{a}	0.68 ± 0.13^{a}	0.23 ± 0.07^{a}
Galacturonic Acid	1.94 ± 0.14^{a}	0.46 ± 0.09^{a}	1.48 ± 0.07^{a}

Different letters indicate significant differences ($p<0.05$) between the monosaccharides of the same sample.

Table 3. Total (TDF), soluble (SDF) and insoluble (IDF) dietary fiber, total phenolic content (TPC) and quality attributes (pH, Aw, hardness and color) of the proposed biscuits: A, control sucrose biscuit; B and C, sucrose biscuits containing alkali-extracted almond shell (AEASF, 3.9% and 15.3%, respectively) as source of dietary fiber; D, control stevia biscuit; and E and F, stevia biscuits containing AEASF (3% and 14.7%, respectively).

	Dietary fiber and antioxidant compounds				Quality attributes					
Biscuits	TDF (%)	SDF (%)	IDF (%)	TPC (mg *GAE/g)	Aw	pH	L*	Color a*	b*	Hardness (†N)
A	3.95±0.45^{a}	36.53±1.19^{b,c}	63.48±1.20^{ab}	0.91±0.13^{a}	0.31±0.01^{c}	6.98±0.12^{b}	100.57±0.45^{d}	1.57±0.10^{b}	27.56±2.05^{c}	40.27±9.42^{a,b}
B	6.26±0.14^{a,b}	30.84±3.81^{b}	69.16±3.80^{b}	1.42±0.14^{b}	0.25±0.04^{b}	7.5±0.08^{c}	56.74±0.85^{b}	12.78±0.54^{c}	12.15±0.90^{c}	58.37±10.97^{b}
C	15.40±2.17^{c}	17.84±2.74^{a}	82.16±2.74^{c}	3.02±0.07^{c}	0.21±0.02^{a}	7.79±0.14^{c}	47.06±1.09^{a}	5.00±0.26^{c}	-2.20±0.22^{a}	158.54±24.01^{d}
D	4.10±0.33^{a}	40.29±2.02^{c}	59.71±2.01^{a}	0.87±0.18^{a}	0.35±0.018^{c}	6.49±0.37^{a}	103.41±0.39^{e}	0.56±0.85^{a}	27.79±2.10^{c}	19.03±1.69^{a}
E	8.12±1.11^{b}	30.92±4.66^{b}	69.08±4.66^{b}	1.34±0.17^{b}	0.32±0.013^{c}	7.04±0.19^{b}	65.82±1.64^{c}	12.20±0.54^{c}	16.37±0.75^{d}	24.69±1.78^{a}
F	17.66±3.35^{c}	19.48±5.15^{a}	80.52±5.16^{c}	2.79±0.07^{c}	0.32±0.02^{c}	7.51±0.09^{c}	46.61±1.37^{a}	7.74±0.24^{d}	1.36±0.51^{b}	92.98±2.75^{c}

*GAE: Gallic Acid Equivalents. †N: Newton. Data are presented as mean ± SD. Different letters indicate significant differences ($p<0.05$) between samples (A-F).

Table 4. Sensory evaluation scores (1 to 9 scale) of biscuits: A, control sucrose biscuit; B and C sucrose biscuits B and C, sucrose biscuits containing alkali-extracted almond shell (AEASF) dietary fiber ("high fiber content", 6.3% and 15.4%, respectively); D, control stevia biscuit, and E and F, stevia biscuits containing AEASF dietary fiber ("high fiber content", 8.12% and 17.7%, respectively).

Biscuits	Color	Flavor	Crispness	Hardness	Mouth feel	Overall acceptability
A	6.8±1.75^{a}	5.8±1.54^{a}	6.5±0.52^{b}	6.1±0.83^{a,b}	6.5±0.52^{b}	7±0.81^{a,b}
B	7.2±1.22^{a}	5.7±1.41^{a}	6.8±0.78^{b}	6.1±1.51^{a,b}	7.8±0.91^{a}	7.6±0.51^{a}
C	5±1.24^{c}	5.7±1.63^{a}	6.9±0.87^{b}	5.4±1.62^{b,c}	6.5±1.50^{b}	7.1±0.99^{a,b}
D	6.3±1.15^{a,b}	6.4±1.17^{a}	5.7±0.82^{c}	5.6±0.48^{b,c}	6.1±0.73^{b}	6.4±0.84^{b,c}
E	6.2±0.78^{a,b}	6.6±1.57^{a}	7.6±0.51^{a}	6.7±0.45^{a}	6.6±1.17^{b}	7.2±0.78^{a,b}
F	5.4±1.07^{b,c}	5.7±1.33^{a}	7±0.66^{a,b}	4.7±0.9^{c}	5±0.94^{c}	6.1±1.19^{c}

Data are expressed as mean ± standard deviation (n=10). In each column, values with different superscript letters are significantly different ($p<0.05$).

Supplementary material

Table 1. Physico-chemical composition of almond shell. Results are expressed as % of total dry material and mg/100g

Analysis	
	g/100g
Moisture	9.39±0.55
Cellulose	32.17±1.32
Hemicelluloses	35.28±1.45
Lignin	28.43±1.61
Proteins	1.64±0.43
Fat	0.46±0.01
Ash	6.77±0.77
	mg/100g
Calcium	2042.5±82.5
Sodium	373±50
Iron	43±16
Magnesium	136.5±3.5
Copper	8.38±1.6
Zinc	0.54±0.00

Data are presented as mean ± SD.
Analyses: moisture [1], ashes [2], minerals [3], fat and protein [4], cellulose, hemicellulose and lignin [5].

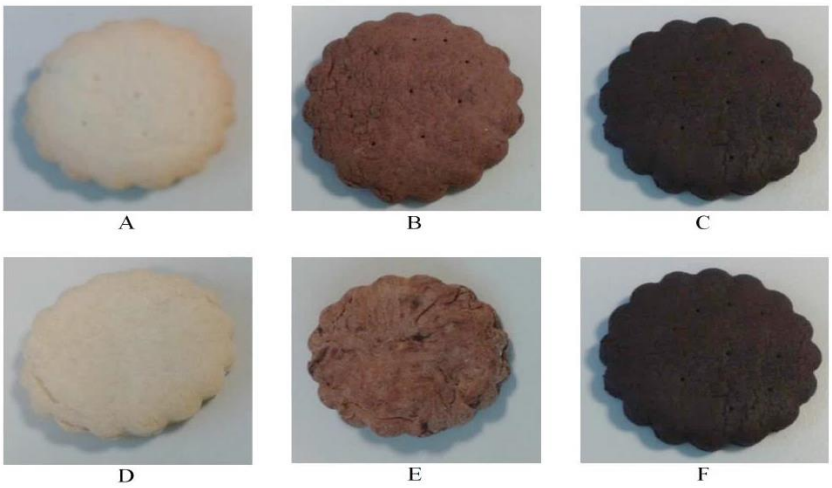

Figure 1. Visual appearance of the novel biscuits: A, control biscuit using sucrose as sweetener; B and C, sucrose biscuits containing alkali-extracted almond shell (AEASF) dietary fiber ("high fiber content", 6.3% and 15.4%, respectively); D, control biscuit using stevia as sweetener; and E and F, stevia biscuits containing AEASF dietary fiber ("high fiber content", 8.12% and 17.7%, respectively).

ANNEX 5

NUTRA ingredients.com

Breaking News on Supplements, Health & Nutrition - Europe

Leftover coffee grounds: Unlocking 6m tonnes of unused antioxidant dietary fibre

By Annie-Rose Harrison-Dunn+ , 24-Aug-2016

Related topics: Antioxidants/carotenoids, Dosage forms & delivery formats, Fibres & carbohydrates, Proteins, peptides, amino acids, Research, Sustainability , New ingredient approvals, Health claims, Functional foods, Baked goods, snacks & sweets

The use of leftover coffee grounds as an antioxidant dietary fibre is a low-cost value-added opportunity for an otherwise waste product, say Spanish researchers.

About two kilograms of wet spent coffee grounds are produced for every kilogram of instant coffee made, which constitutes to about six million tonnes each year globally.

While uses in biofuels, composts, animal feed and enzymes have been explored, researchers from Madrid say there is now an increasing interest in food and health uses for the by-product.

According to their paper in the journal *Food Chemistry*, spent coffee grounds from industrial instant coffee are a promising natural source of antioxidant insoluble dietary fibre, proteins, essential amino acids and low glycaemic sugars, which could be used in bakery products in the future.

"These food formulations might be destined to people with reduced energetic intake and particular requirements," wrote the team from the Autonoma University of Madrid (UAM) and the Spanish National Research Council (CSIC).

"The application of spent coffee grounds which we propose represents a value-added opportunity for coffee by-products utilisation at a very low cost."

One of the researchers behind the paper, Dr Maria Dolores del Castillo, told us the ingredient's antioxidant potential added another attractive benefit to the fibre source.

"They are bioavailable antioxidants as they are using the dietary fibre as transport to the gastrointestinal tract," said Dr Dolores del Castillo, senior scientist and head of the Food Bioscience Group at the UAM's Institute of Food Science Research (CIAL).

"This should be cheaper [than other fibre sources] because it is a very abundant by-product."

She said one manufacturer of coffee reported producing two tonnes of the grounds a day.

The paper comes as part of the research project SUSCOFFEE, which was backed by a €185,000 grant from the Spanish Ministry of Economy and Competitiveness.

Her team was now seeking companies interested in the development and commercialisation of the food formulation under patent license.

"Its use as healthy food ingredient is a contribution to the bioeconomy and the reduction of the environmental impact of the coffee processing. The intake of antioxidants and particularly of antioxidant dietary fibre is suggested for the prevention of chronic diseases and the improvement of the gastrointestinal health," said Dolores del Castillo.

Testing the recipe

Using grounds from Spanish private label coffee maker Prosol, the researchers made up six different sugar-free sample biscuits with varying amounts of the grounds ranging from 3.5–4.4%.

Each prototype would be able to make the nutritional claims 'source of fibre' at more than 3 g fibre per 100 g biscuit or 'high fibre content' with over 6 g fibre per 100 g biscuit in the EU.

Stevia was also used for sweetness as well as Beneo-Orafti-donated oligofructose as an enhancer of glucose tolerance and prebiotic source.

Dolores del Castillo said taste of adding the grounds – which leads to a similar appearance in the biscuits to chocolate chips – was masked by these two ingredients.

They concluded that up to 4% of the grounds could be used as a food ingredient in solid foods like biscuits without affecting the quality of the product.

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About 70% of a tasting panel 'accepted' the biscuits using the grounds and they said the biscuits tapped into consumer interest in healthier alternatives.

"Consumers are concerned about caloric content and glycaemic index (GI) of the food as well as balanced nutrition comprising dietary fibre content," they wrote.

"The benefits of low GI diets extend beyond weight loss and have favourable effects on obesity-related diseases such as type 2 diabetes."

Super fibre?

Caffeine and the antioxidant chlorogenic acid (CGA) are the major bioactive compounds of coffee.

The freeze-dried spent coffee grounds 200 mg caffeine per 100 g and 10 mg CGA per 100 g.

Yet said Dolores del Castillo said this coffee content was minimal working out at about 8 mg per 100 g of dough. This is far below that which would require an on-pack warning for pregnant women and children in the EU.

The grounds also contained a *"significant"* amount of proteins as well as essential amino acids glutamic acid, threonine, aspartic acid and leucine.

Source: *Food Chemistry*

Published online ahead of print, **http://dx.doi.org/10.1016/j.foodchem.2016.07.173**

"Use of spent coffee grounds as food ingredient in bakery products"

Authors:N. Martinez-Saez, A. Tamargo García, I. Domínguez Pérez, M. Rebollo-Hernanz, M. Mesías, F. J. Morales, M. A. Martín-Cabrejas and M. Dolores del Castillo

JANUARY 30, 2017 PUBLISHED IN APPLIED RESEARCH EMAIL PRINT

Coffee by-product may be used a potential ingredient in bakery products

Spent coffee grounds (SCG) are a by-product (45%) of coffee beverage preparation and instant coffee manufacturing. Currently it is reported that SCG could be used in several applications including biofuels, composts, animal feed, biosorbents and enzymes.

A study published in the journal **Food Chemistry** has investigated whether SCG could be used as a food ingredient in bakery products, as a source of antioxidant dietary fibre. Del Castillo et al. investigated whether the by-product could

be used to produce biscuits with high nutritional and sensory quality and potentially help to reduce the risk of chronic diseases including obesity and diabetes. The team also investigated the safety of using SCG as a food ingredient, noting that the maillard reaction can produce health promoting effects as well as potentially harmful effects including acrylamide and furan.

The team from the Spain produced 6 free sugar biscuits prepared with wheat flours and sunflower oils, with sucrose being replaced by stevia and/or malitol, and SGC added in amounts ranging from 3.5% to 4.4% in order to achieve the nutritional claim "source of fibre" or "high fibre content". Del Castillo et al. purchased three commercial biscuits, two of which were used in the sensory analysis (involving 26 untrained panellists, who rated the 6 biscuits for colour, texture, taste and overall acceptance) as they contain a similar composition to the SCG biscuits, the third was used as "a control of the average formation of potential harmful compounds during food processing." Using the biscuit named in the study as B2 (containing 4.24g of SCG, and used due to its high nutritional and sensorial quality), and the commercial control biscuit, the scientists evaluated proteins, soluble free amino acids, fructosamine, advanced glycation end products, and also microbiological quality and food processing contaminants. Data was also collected on physiochemical characterisation (including moisture, total protein, dietary fibre, total phenolic, overall antioxidant capacity), and thermal stability.

The Researchers. report that SCG from the instant coffee process was "found to be a natural source of antioxidant insoluble dietary fibre, essential amino acids with low glycaemic sugar, resistant to thermal food processing condition and digestion process and totally safe. Therefore, SCG could be incorporated as food ingredients in bakery products for human consumption." Regarding acceptability, the panellists rated the innovative biscuits containing oligofructose as not being significantly different to the commercial biscuits. The scientists also report that the three biscuits containing SCG at levels of 4.24 g, 3.64 g and 3.5 g, might be included inside the "high fibre" nutritional claim.

In conclusion, the scientist state "SCG (4% w/w) can be used directly as a food ingredient in solid food as biscuits without affecting the conventional food preparation and final quality of the product. These food formulations might be destined to people with reduced energetic intake and particular requirements."

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Brief Curriculum Vitae

Educational degrees

- **Sept 2011 – Sept 2012**: Master's degree in Agricultural Chemistry and Novel Foods. Department of Agrochemistry, School of Science, Autonomous University of Madrid (UAM).
- **Sept 2009 – Jun 2011**: Bachelor's degree in Food Science and Technology. UAM.
- **Sept 2006 – Jun 2009**: Three-year Bachelor's degree in Human Nutrition. UAM.

Research student grants

- **Sept 2013 – Sept 2017:** Pre-doctoral Student Fellowship granted by the UAM. Workplace: Food Bioscience Group at the Institute of Food Science Research (CIAL, UAM-CSIC), Madrid, Spain.
- **Oct 2011 – Oct 2013:** Post-graduate Student Fellowship granted by the UAM. Workplace: Food Bioscience Group at the CIAL (UAM-CSIC), Madrid, Spain.
- **Oct 2010 – Oct 2011:** Research Student Fellowship granted by the Spanish Ministry of Education. Workplace: Agrifood Chemistry Group at the CIAL (UAM-CSIC), Madrid, Spain.
- **Jul 2010 – Sept 2010**: Research Student Fellowship granted by the Spanish National Research Council (CSIC). Workplace: Institute of Science and Technology Food and Nutrition (ICTAN-CSIC).

Participation in research projects:

- SUSCOFFEE, AGL2014-57239-R, MINECO. "Producción y consumo sostenibles del café: validación de subproductos como ingredientes alimentarios".
- INFOGEST Cost Action oc-2010-1-7087. Improving health properties of food by sharing our knowledge on the digestive process.
- NATURAGE, AGL2010-17779, MINECO. "Nuevos ingredientes antiglicantes: Mecanismos de acción y aplicaciones en alimentación y salud".
- A2/036996/11, AECID. "Obtención de compuestos bioactivos de subproductos de origen vegetal. Aplicación en alimentación y salud".

Research experience

- **Dec 2012 – present:** PhD student at the Food Bioscience Group at the Institute of Food Science Research (CIAL, UAM-CSIC), Madrid, Spain.
- **Sept 2016 – Dec 2017:** Visiting PhD student at the Nephrology Division, Icahn School of Medicine at Mount Sinai Hospital, New York, USA. Supervisor: Professor Dr. Jaime Uribarri.

- **Apr 2015 – Jul 2015**: Visiting PhD student at the Nutritional and Physiological Chemistry Group, Faculty of Chemistry, University of Vienna, Austria. Supervisor: Professor Dr. Veronika Somoza.

— *Research articles*: published (5), submitted (4).
— *Book chapters*: 5 (3 published, 2 in press)
— *Participation in congresses*: 11 (3 oral communications and 8 posters)
  - *Member of the Collaborating Committee of the IX Congress CyTA-CESIA.*

Academic experience

- **2017:** Practical classes of the subject "Sensorial Analysis" (Bachelor's Degree in "Food Sciences", 3rd course) at the UAM (36 h).
- **2015:** Practical classes of the subject "Instrumental and Sensorial Analysis of Foods" (Bachelor's Degree in "Human Nutrition and Dietetics", 2nd course) at the UAM (36 h).
- **2014:** Practical classes of the subject "Food Products" (12 h) (Bachelor's Degree in "Food Sciences", 2nd course) and "Instrumental and Sensorial Analysis of Foods" (36h) (Bachelor's Degree in "Human Nutrition and Dietetics", 2nd course) at the UAM.

Merits

- Best Oral Communication Awards:
  — "Reduction of After-Taste in Innovative Biscuits Formulations Made with Stevia". 7th World Convention on Stevia: Stevia Tasteful - Science, Formulation & Extraction: The Subtle Balance. 11-12th June, 2015. Berlin, Germany.
  — "Aplicación de los marros de café como fuente natural de fibra antioxidante". I Jornadas Científicas CIALForum, 5th June, 2014, Madrid. Spain.
- Graduation with Honors**:**
  — Bachelor's degree in "Food Science and Technology", UAM (2009-2011).
  — Three-year Bachelor's degree in "Human Nutrition and Dietetic", UAM (2006-2009).

Overview of the PhD training activities

Participation in congresses

1. "*In vitro* formation of carboxymethyl-lysine (CML) under physiological digestion conditions". **N. Martinez-Saez**, B. Fernandez-Gomez, C. Weijing, M.D. del Castillo, J. Uribarri. 5th International Conference on Food Digestion. 4-6th April 2017, Rennes, France. Oral presentation.
2. "Antioxidant coffee dietary fiber for gastrointestinal health and diabetes". K. Vázquez Sánchez, **N. Martinez-Saez**, M. D del Castillo, R. Campos Vega. 20th International Conference of Functional Food Center. Functional and Medical Foods for Chronic Diseases: Bioactive Compounds and Biomarkers. 22-23rd September 2016, Harvard Medical School, Boston, USA. Oral presentation.
3. "Innovative biscuits for a better tolerance to carbohydrates". **N. Martinez-Saez**, M. A. Martín-Cabrejas, M.D. del Castillo. 17th International Congress of Dietetics. 7-10th September, 2016, Granada, Spain. Oral presentation.
4. "Determinación de la bioaccesibilidad de productos de la reacción de Maillard y antioxidantes en café usado (Coffea arabica L.)". K, Vázquez Sánchez, M. D. del Castillo, **N., Martinez-Saez**, R., Campos Vega. Congreso Internacional de Investigación e Innovación. 21-22nd April, 2016, Guanajuato, Mexico. Poster.
5. "Effect of new biscuit formulations in the release of satiety hormones". **N. Martinez-Saez**, C.M. Hochkogler, V. Somoza, M.D. del Castillo. 7th International Simposium of Food Development and Innovation: "Challenges, progress and innovation in food processing", 7-9th October, 2015, LATU, Montevideo, Uruguay. Poster.
6. "Reduction of after-taste in innovative biscuit formulations made with stevia". **N. Martinez-Saez,** M.D. del Castillo. 7th World Convention on Stevia: Stevia Tasteful 2015 - Science, Formulation & Extraction: The Subtle Balance. 11-12th June, 2015, Berlin, Germany. Oral presentation.
7. "Feasability of spent coffee grounds as dietary fibre". **N. Martinez-Saez,** M. Ullate, M.A., Martín-Cabrejas, M.D. del Castillo. II Simposio Científico Internacional para la Innovación en la Industria Marina y Alimentaria, 15-16th September, 2014, Vigo, Spain. Poster.
8. "Aplicación de los marros del café como fuente de fibra antioxidante". **N. Martinez-Saez,** M. Ullate, M.A., Martín-Cabrejas, M.D. del Castillo. CIALFORUM, Madrid, España. 5th June, Madrid, Spain. Oral presentation.

9. "A novel antioxidant beverage for body weight control based on coffee silverskin". **N. Martinez-Saez,** M. Ullate, M.A., Martín-Cabrejas, P. Martorell, S.R. Genovés, M.D. del Castillo. Second International Congress on Cocoa and Tea. 9-11th October, 2013, Naples, Italy. Oral presentation.
10. "Innovative alternative use of coffee by-products". M. Ullate, B. Fernández-Gomez, **N. Martinez-Saez,** M.D. del Castillo. Second International Congress on Cocoa and Tea. October 9-11th, 2013, Naples, Italy. Poster.
11. "Mitigation of food processing contaminants by alternative sugar substitutes". M.D. del Castillo, **N. Martinez-Saez,** E. García-Serna, **F.J. Morales.** EUROFOODCHEM XVII. 7-10th May, 2013, Istanbul, Turkey. Poster.

Participation in teacher training programs

Practical classes at the UAM of the following subjects :

2017 - "Sensorial Analysis" (Bachelor's Degree in "Food Sciences", 3rd course)

2015 - "Instrumental and Sensorial Analysis of Foods" (Bachelor's Degree in "Human Nutrition and Dietetics", 2nd course).

2014 - "Food Products" (Bachelor's Degree in "Food Sciences", 2nd course) and "Instrumental and Sensorial Analysis of Foods" (36h) (Bachelor's Degree in "Human Nutrition and Dietetics", 2nd course).

Training courses

1) **II Technical Training School**: "How to use the consensus model, calibrate the digestive enzymes". INFOGEST Cost Action. 19-23rd January, 2015, Faculty of Pharmacy, University of Granada, Spain.
2) **I Jornadas de Ciencia y Gastronomía**: De la Ciencia a la Innovación. FACYRE. 22-23rd September, 2014. CIAL, Madrid, Spain.
3) **Training course for working with experimental animals**: Category B. BINAEX. 21st April -9th June, 2014. Accredited by the "Área de Protección Animal de la DG de Medio Ambiente de la Comunidad de Madrid por Resolución 10/114426,9/14 de fecha 13/05/2014".
4) **II Training School:** "Food Digestion and Human Health". INFOGEST Cost Action. 24-28th March, 2014, at the Food Science Institute, Budapest, Hungary.